CERTIFICATE OF ELECTRONIC TRANSMISSION
37 C.F.R. § 1.8

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July 25, 2007
Date

David L. Parker

Docket No.: AH-CLFR:092US
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Rosenblum *et al.*

Application No.: 09/320,156

Filed: May 26, 1999

Art Unit: 1643

For: IMMUNOTOXINS DIRECTED AGAINST C-ERBB2 (HER-2/NEU) RELATED SURFACE ANTIGENS

Examiner: Canella, K.A.

CORRECTED APPEAL BRIEF

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Appellants hereby submit this Corrected Appeal Brief to the Board of Patent Appeals and Interferences in response to the Communication mailed June 29, 2007. In that Communication, it was stated that the listing of the claims was incorrect. The present Brief should have the correct set of claims.

The original Notice of Appeal was filed for this case on November 22, 2002, and the original Brief was filed on January 13, 2003. On August 18, 2005, an Order Remanding Appeal to Examiner was mailed, and on June 14, 2006, the Examiner issued an Office Action. A further Appeal Brief was filed September 14, 2006, and an Examiner's Answer was filed on December 1, 2006. A Reply Brief was filed January 29, 2007 along with a request for oral argument and appropriate fees.

I. REAL PARTY IN INTEREST

The real party in interest is the assignee, Research Development Foundation.

II. RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences.

III. STATUS OF THE CLAIMS

Claims 1-21 were originally filed. Claims 1-14 and 20-21 are withdrawn. Claim 18 was canceled. Claims 15-17 and 19 are under examination and are the subject of appeal.

IV. STATUS OF AMENDMENTS

There are no pending amendments.

V. SUMMARY OF THE CLAIMED SUBJECT MATTER

The present invention generally concerns compositions related to conjugates or fusion proteins of tumor necrosis factor (TNF) to scFv-23, a single chain antibody that exhibits binding specificity for an extracellular epitope of c-erbB-2, as represented in claims 15 and 16, respectively, and as described in the specification at least on page 5, line 13-page 6, line 2. In particular aspects the invention further concerns the fusion protein being recombinantly produced by fusing a gene encoding the single chain antibody to a gene encoding TNF, as represented in claim 17, and as described in the specification at least on page 5, line 13-page 6, line 2. In further aspects of the invention, the conjugate composition is comprised in a pharmaceutically acceptable vehicle, as represented in claim 19, and as described on page 48, lines 5-9.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

Claims 15 and 19 are rejected under 35 U.S.C. §103(a) as allegedly being unpatentable over King *et al.* (U.S. Patent No. 5,587,458; "King") in view of Rosenblum *et al.* (Cancer Communications, 1991, Vol. 3, pp. 21-27; "Rosenblum").

Claims 15-17 and 19 are rejected under 35 U.S.C. §103(a) as allegedly being unpatentable over King and Rosenblum and further in view of Gillies (U.S. Patent No. 5,650,150).

VII. ARGUMENT

A. Substantial Evidence Required to Uphold the Examiner's Position

As an initial matter, Appellants note that findings of fact and conclusions of law by the U.S. Patent and Trademark Office must be made in accordance with the Administrative

Procedure Act, 5 U.S.C. § 706(A), (E), 1994. *Dickinson v. Zurko*, 527 U.S. 150, 158 (1999). Moreover, the Federal Circuit has held that findings of fact by the Board of Patent Appeals and Interferences must be supported by "substantial evidence" within the record. *In re Gartside*, 203 F.3d 1305, 1315 (Fed. Cir. 2000). In *Gartside*, the Federal Circuit stated that "the 'substantial evidence' standard asks whether a reasonable fact finder could have arrived at the agency's decision." *Id.* at 1312.

Accordingly, it necessarily follows that an Examiner's position on Appeal must be supported by "substantial evidence" within the record in order to be upheld by the Board of Patent Appeals and Interferences.

B. Issues under 35 U.S.C. §103(a)

1. King in view of Rosenblum

Claims 15 and 19 are rejected under 35 USC §103(a) as being unpatentable over King *et al.* (U.S. Patent No. 5,587,458; "King") and Rosenblum *et al.* (Cancer Communications, 1991, vol. 3, pp. 21-27; "Rosenblum"). Appellants respectfully disagree.

There is no motivation or suggestion to combine Rosenblum and King, and therefore the Examiner has failed to make a *prima facie* case of obviousness. King concerns a conjugate of single chain anti-erbB-2 antibodies to a cytotoxic moiety, and Rosenblum describes conjugation of TNF to a murine antibody, ZME-018, which binds an epitope of a high molecular weight antigen, gp240, found on the surface of some melanoma cells. King makes no mention or suggestion to employ TNF as a cytotoxic moiety and, more importantly, provides no motivation to employ TNF. In column 8, lines 47-54, King teaches the skilled artisan to employ antibodies attached to cytotoxic moieties, such as radioactive materials, anti-cancer drugs, anti-metabolies, inhibitors of protein synthesis, and agents that bind DNA. None of these are cytokines, and there is no suggestion to use cytokines. King explicitly provides a list of usable moieties and yet

excludes cytokines in the list, so the skilled artisan would have no motivation or suggestion to employ cytokines, and certainly not to utilize TNF specifically.

In fact, King also provides no motivation or suggestion to combine with another reference, and certainly not Rosenblum, because King heralds the use of conjugation of the antibody e23 to the exemplary *Pseudomonas* exotoxin A variant PE40 and derivatives thereof as cytotoxic moieties (see at least column 8, line 47-column 9, line 11 and column 18, line 60-column 21, line 19), so there would be no motivation to utilize any reagents other than what is taught by King as being successful or as alternatives thereto. Given that King reports success with the technology, one of skill in the art would find no motivation to expend time and resources to alter successful protocols.

Appellants note that the person of ordinary skill in the art is an objective legal construct presumed to think along conventional lines without undertaking to innovate, whether by systematic research or by extraordinary insights. *Life Technologies, Inc. v. Clontech Laboratories, Inc.*, 224 F.3d 1320, 56 U.S.P.Q.2d 1186 (Fed. Cir. 2000), citing *The Standard Oil Co. v. American Cyanamid Company*, 774 F.2d 448, 227 U.S.P.Q. 293 (Fed. Cir. 1985), which states the following:

> The statutory emphasis is on a person of ordinary skill. Inventors, as a class, according to the concepts underlying the Constitution and the statutes that have created the patent system, possess something--call it what you will--which sets them apart from the workers of ordinary skill, and one should not go about determining obviousness under §103 by inquiring into what patentees (*i.e.*, inventors) would have known or would likely have done, faced with the revelations of references. **A person of ordinary skill in the art is also presumed to be one who thinks along the line of conventional wisdom in the art and is not one who undertakes to innovate,** whether by patient, and often expensive, systematic research or by extraordinary insights, it makes no difference which (emphasis added).

Even if one were motivated to combine the teachings of King with another reference, which Appellants do not acquiesce, there is no motivation or suggestion to specifically combine King with Rosenblum. Rosenblum similarly reports success with his technology (see at least right column or p. 23-right column of p. 25; "impressive growth inhibition effects" (p. 25, right column)). Also, King was filed two years after Rosenblum, and yet King chose not to describe or even suggest conjugating scFv23 to TNF even though a variety of cytotoxic moieties were listed in King (see at least column 8, lines 47-54). In addition, by teaching use of a monoclonal antibody, Rosenblum teaches away from using a single chain antibody. By describing conjugation of TNF to an antibody specific for a melanoma, Rosenblum at most might lead one of skill in the art to crosslink TNF to an alternative monoclonal antibody specific for a surface epitope of a melanoma cell. Even if Appellants were to acquiesce that Rosenblum provides motivation to utilize antibodies other than ZME-018 for delivery of TNF, which they do not, there is no guidance or suggestion in King which of the plethora of antibodies in the art to select from.

Appellants thus contend that the Examiner has engaged in an improper hindsight reconstruction, picking and choosing from King and Rosenblum that differ in their teachings. "One cannot use hindsight reconstruction to pick and choose among isolated disclosures in the prior art to depreciate the claimed invention." *In re Fina*, 5 USPQ2d 1596, 1600 (Fed. Cir. 1988).

Appellants also respectfully remind the Examiner that section 103 requires consideration of the claimed invention "as a whole." This "as a whole" requirement prevents evaluation of the invention part by part, in hindsight. *Envtl. Designs, Ltd. v. Union Oil Co.*, 713 F.2d 693, 698 (Fed. Cir. 1983). Without this requirement, an obviousness assessment could break an invention

into its component parts (in specific embodiments, *e.g.*, conjugation of scFv23 antibody and TNF), then find a prior art reference containing the component parts (*e.g.*, conjugation of scFv23 as described by King and TNF as described by Rosenblum), and on that basis alone declare the invention obvious. The courts have refused to act on this type of hindsight reasoning, which uses the invention as a roadmap to find its prior art components. This type of analysis discounts the value of novel selection inventions. Thus, the courts have required that an Examiner must show some suggestion or motivation, *excluding the invention itself*, to make the new combination. See *In re Rouffet*, 149 F.3d 1350, 1355-56 (Fed. Cir. 1998); *In re Lee* 277 F. 2d 1338, 61 USPQ 2d 1430 (Fed. Cir. 2002); and *c.f. Ruiz v. A.B. Chance Co.*, F.3d 1270 (Fed. Cir. 2004) (emphasis added).

Furthermore, on page 3 of the Action, the Examiner states that one of skill in the art would have been motivated to substitute TNF for the cytotoxic moiety employed in King. As noted in MPEP 2144.03 and in keeping with *In re Zurko* (258 F.3d 1385, 59 USPQ2d 1697 (Fed. Cir. 2001)), an assessment of basic knowledge and common sense that is not based on any evidence in the record lacks substantial evidence support, and the Examiner has provided no evidentiary support for the assertion that one of skill in the art would be motivated to change the cytotoxic moiety of King to TNF. The Examiner must provide specific factual findings predicated on sound technical and scientific reasoning to support his or her conclusion of common knowledge. *In re Chevenard*, 139 F.2d 713, 60 USPQ 241 (CCPA 1943). *In re Soli*, 317 F. 2d 941, 945-946, 137 USPQ 797, 800 (CCPA 1963). Moreover, if the Appellant adequately traverses the Examiner's assertion of official notice, the Examiner must provide documentary evidence in the next action if the rejection is to be maintained. See 37 CFR §1.104 (c)(2) and *Zurko*, 258 F.3d at 1386, 59 USPQ2d at 1697. If the Examiner is relying on personal

knowledge to support the finding of what is known in the art, the Examiner must provide an affidavit or declaration setting forth specific factual statements and explanation to support the finding. See 37 CFR §1.104(d)(2).

Official notice unsupported by documentary evidence should only be taken by the examiner where the facts asserted to be well-known, or to be common knowledge in the art, are capable of instant and unquestionable demonstration as being well-known. As noted by the court in *In re Ahlert*, 424 F.2d 1088, 1091, 165 USPQ 418, 420 (CCPA 1970), the notice of facts beyond the record which may be taken by the examiner must be "capable of such instant and **unquestionable demonstration as to defy dispute**" (citing *In re Knapp* Monarch Co., 296 F.2d 230, 132 USPQ 6 (CCPA 1961)) (emphasis added).

Appellants assert that the claims are not unpatentable over King in view of Rosenblum at least because there is no motivation or suggestion to combine the references, and Appellants respectfully request reversal of the rejection.

2. King and Rosenblum in view of Gillies

Claims 15-17 and 19 are rejected under 35 USC §103(a) as being unpatentable over King in view of Rosenblum and further in view of Gillies *et al.* (U.S. Patent No. 5,650,150; "Gillies"). Appellants respectfully disagree.

a) Claims 15 and 19

As argued in Section VII.B.1. of this Brief, claims 15 and 19 are not unpatentable over the combination of King and Rosenblum given that neither King nor Rosenblum provide suggestion or motivation to combine with each other. It follows, then, that there can be no motivation to combine these references with any other reference, including Gillies. Furthermore, the Examiner cites Gillies as an obvious combination with King and Rosenblum because Gillies teaches a recombinant fusion of TNF-alpha to the heavy chain variable region of an antibody, yet

by teaching a recombinant fusion of TNF to scFv23 Gillies teaches away from the conjugate nature of the subject matter of claims 15 and 19.

b) Claims 16 and 17

The Examiner alleges that claims 15-17 and 19, but particularly claim 16, are further obvious in view of Gillies because Gillies concerns making recombinant fusion of TNF-alpha to the heavy chain variable region of an antibody. As discussed above, the claims are not unpatentable over the combination of King and Rosenblum because there is no motivation or suggestion to combine King and Rosenblum with each other; therefore, there can be no motivation to combine these references with any other reference, including Gillies.

Appellants assert that even if there were motivation or suggestion to combine King with Rosenblum, which Appellants do not acquiesce, there is furthermore no motivation to combine King and Rosenblum with Gillies. Gillies concerns recombination of TNF with a heavy chain variable region of an antibody. Having an earlier filing date than King and Rosenblum, the skilled artisan would be aware of Gillies' techniques to employ recombinant fusions, and yet neither King nor Rosenblum chose to utilize such methods in their working Examples. Therefore, one of skill in the art would similarly not be motivated to employ recombination fusions over the preferred conjugation techniques of King and Rosenblum.

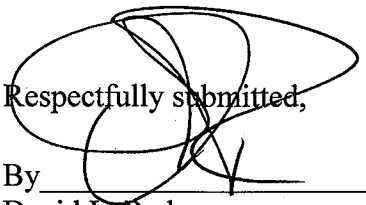
Appellants respectfully request reversal of the rejection.

VIII. CONCLUSION

Appellants have provided arguments that overcome the pending rejection. Appellants respectfully submit that the Office Action's conclusions that the claims should be rejected are unwarranted. It is therefore requested that the Board overturn the Action's rejections.

Dated: July 25, 2007

Respectfully submitted,



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APPENDIX 1

CLAIMS ON APPEAL

15. A composition comprising a conjugate of tumor necrosis factor to a single chain antibody exhibiting binding specificity for an extracellular epitope of c-erbB-2 protein, wherein said single chain antibody is scFv-23.

16. The composition of Claim 15, wherein said conjugate is a fusion protein between said single chain antibody and tumor necrosis factor.

17. The composition of Claim 15, wherein said conjugate is recombinantly produced by fusing a gene encoding said single chain antibody to a gene encoding tumor necrosis factor.

19. The pharmaceutical composition, comprising the composition of claim 15 and a pharmaceutically acceptable vehicle.

APPENDIX 2

EVIDENCE APPENDIX

Exhibit 1. King *et al.* (U.S. Patent No. 5,587,458) made of record in the Office Action mailed June 14, 2006

Exhibit 2. Rosenblum *et al.* (Cancer Communications, 1991, Vol. 3, pp. 21-27) made of record in the Office Action mailed April 26, 2000

Exhibit 3. Gillies (U.S. Patent No. 5,650,150) made of record in the Office Action mailed June 14, 2006

Exhibit 1

US005587458A

United States Patent [19]

King et al.

[11] Patent Number: 5,587,458

[45] Date of Patent: Dec. 24, 1996

[54] **ANTI-ERBB-2 ANTIBODIES, COMBINATIONS THEREOF, AND THERAPEUTIC AND DIAGNOSTIC USES THEREOF**

[75] Inventors: **C. Richter King**; **Philip G. Kasprzyk**, both of Washington, D.C.; **Robert E. Bird**, Rockville, Md.

[73] Assignee: **Aronex Pharmaceuticals, Inc.**, The Woodlands, Tex.

[21] Appl. No.: **61,092**

[22] Filed: **May 14, 1993**

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 906,555, Jun. 30, 1992, which is a continuation-in-part of Ser. No. 772,270, Oct. 7, 1991, abandoned.

[51] **Int. Cl.**[6] **C07K 16/00**; C07K 16/46; C07K 16/28

[52] **U.S. Cl.** **530/387.3**; 530/387.7; 530/388.22; 530/389.7; 530/391.3; 530/391.7; 436/501; 436/512

[58] **Field of Search** 530/387.1, 387.3, 530/387.7, 388.1, 388.22, 388.8, 389.7, 391.1, 391.3, 391.7, 388.85, 409, 866, 367, 370, 300, 350; 424/86.91, 130.1, 133.1, 134.1, 135.1, 138.1, 141.1, 143.1, 155.1, 156.1, 192.1, 193.1; 436/501, 502, 536; 425/7.92, 7.1, 183

[56] **References Cited**

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Lenz et al. Gene 87:213–218 (1990).

Primary Examiner—Donald E. Adams
Attorney, Agent, or Firm—C. Steven McDaniel; Conley, Rose & Tayon, P.C.

[57] **ABSTRACT**

The present invention relates to novel antibodies, in particular monoclonal and single chain antibodies derived therefrom which specifically bind to erbB-2, as well as diagnostic and therapeutic uses thereof. The present invention also relates to a combination of at least two erbB-2 specific antibodies which are capable of preventing and treating human malignancies wherein the malignant cells overexpress $gp185^{erbB-2}$. The monoclonal antibodies of the combination preferably recognize different epitopes of the gp185 expression product of erbB-2, therefore, the antibodies do not cross react with each other. Preferably, the combination will provide for synergistic decrease in the expression of the erbB-2 gene product.

12 Claims, 10 Drawing Sheets

FIG. 1A

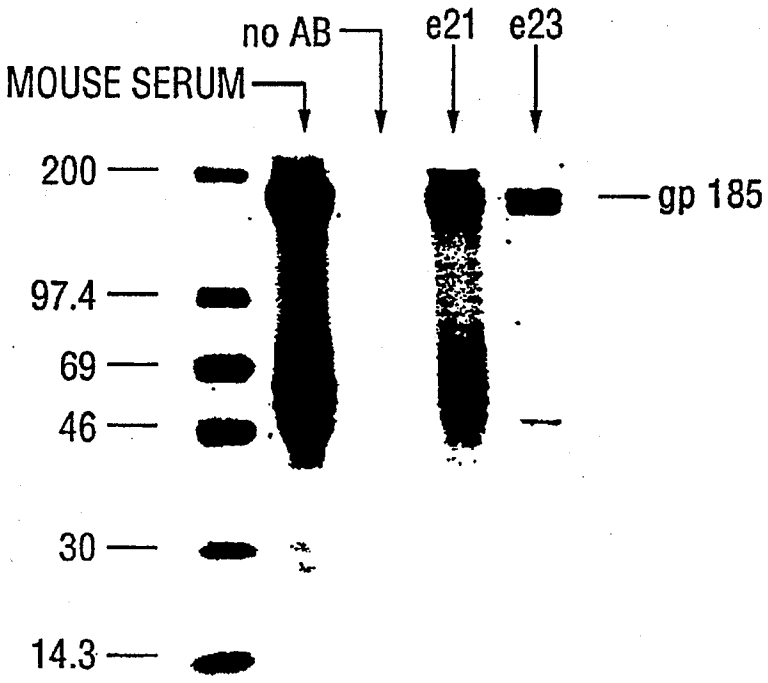
no AB
e21
e23
MOUSE SERUM
200
97.4
69
46
30
14.3
gp 185

FIG. 1B

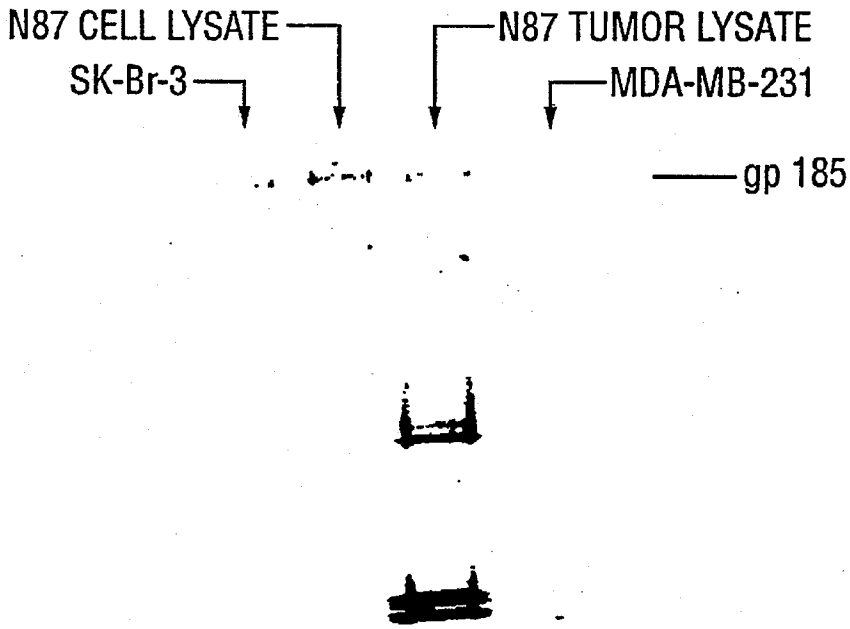
N87 CELL LYSATE
SK-Br-3
N87 TUMOR LYSATE
MDA-MB-231
gp 185

FIG. 1C

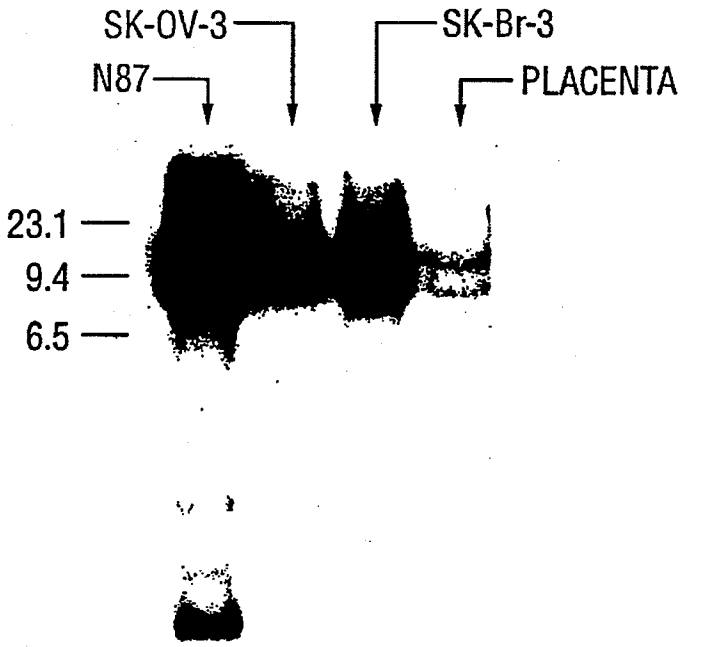
SK-OV-3
SK-Br-3
N87
PLACENTA
23.1
9.4
6.5

FIG. 2

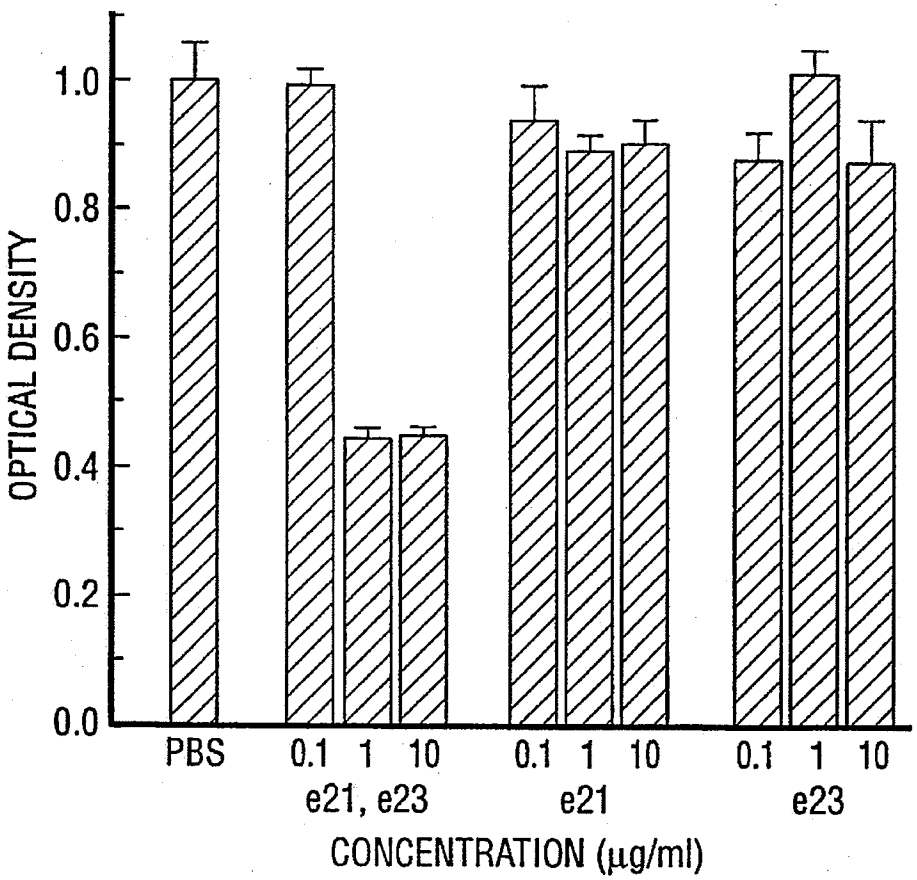
OPTICAL DENSITY
1.0
0.8
0.6
0.4
0.2
0.0
PBS
0.1 1 10
e21, e23
0.1 1 10
e21
0.1 1 10
e23
CONCENTRATION (μg/ml)

FIG. 3A

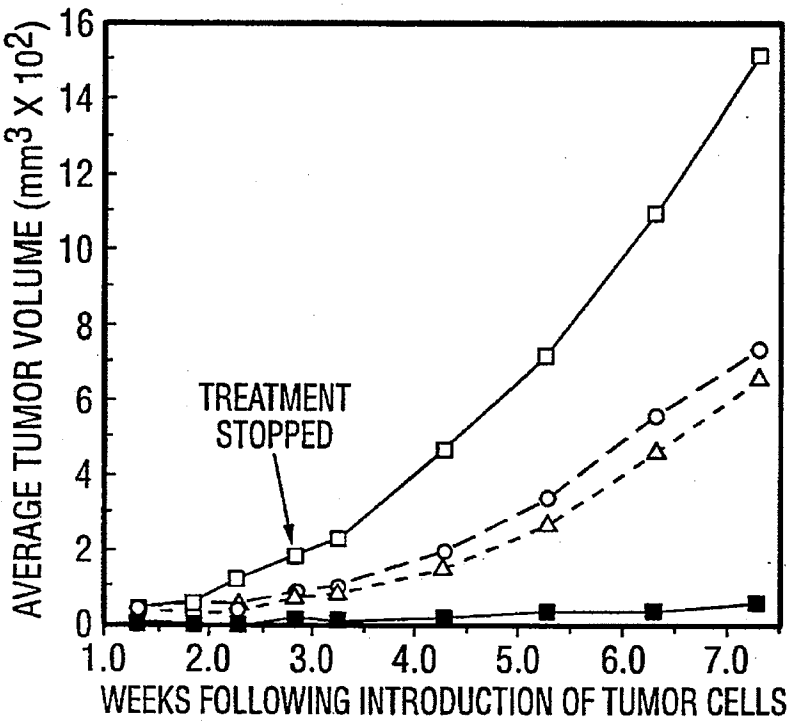
AVERAGE TUMOR VOLUME (mm^3 X 10^2)
16
14
12
10
8
6
4
2
0
TREATMENT STOPPED
1.0
2.0
3.0
4.0
5.0
6.0
7.0
WEEKS FOLLOWING INTRODUCTION OF TUMOR CELLS

FIG. 3B

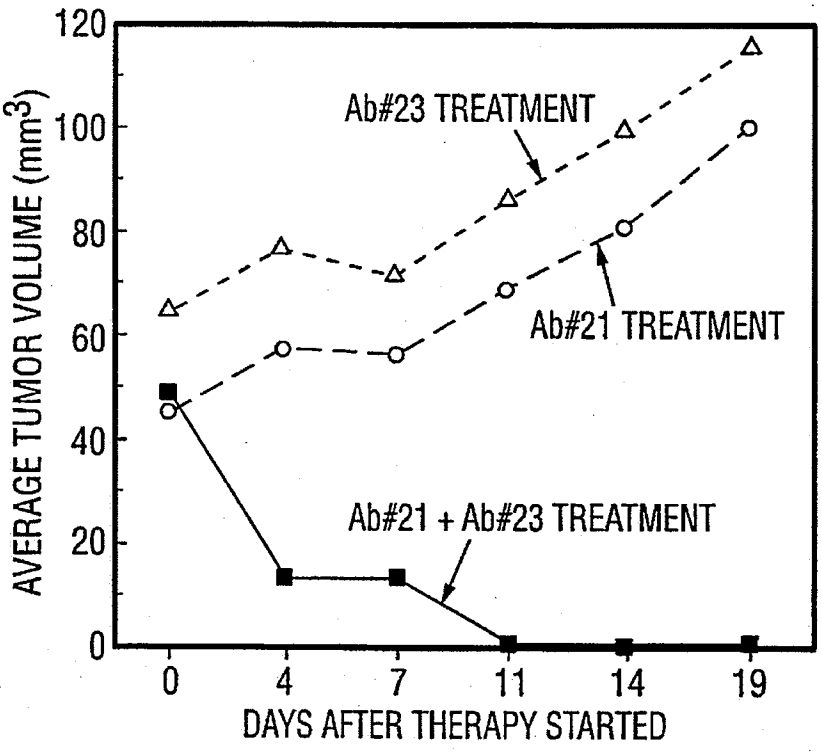
AVERAGE TUMOR VOLUME (mm^3)
120
100
80
60
40
20
0
Ab#23 TREATMENT
Ab#21 TREATMENT
Ab#21 + Ab#23 TREATMENT
0
4
7
11
14
19
DAYS AFTER THERAPY STARTED

FIG. 4A

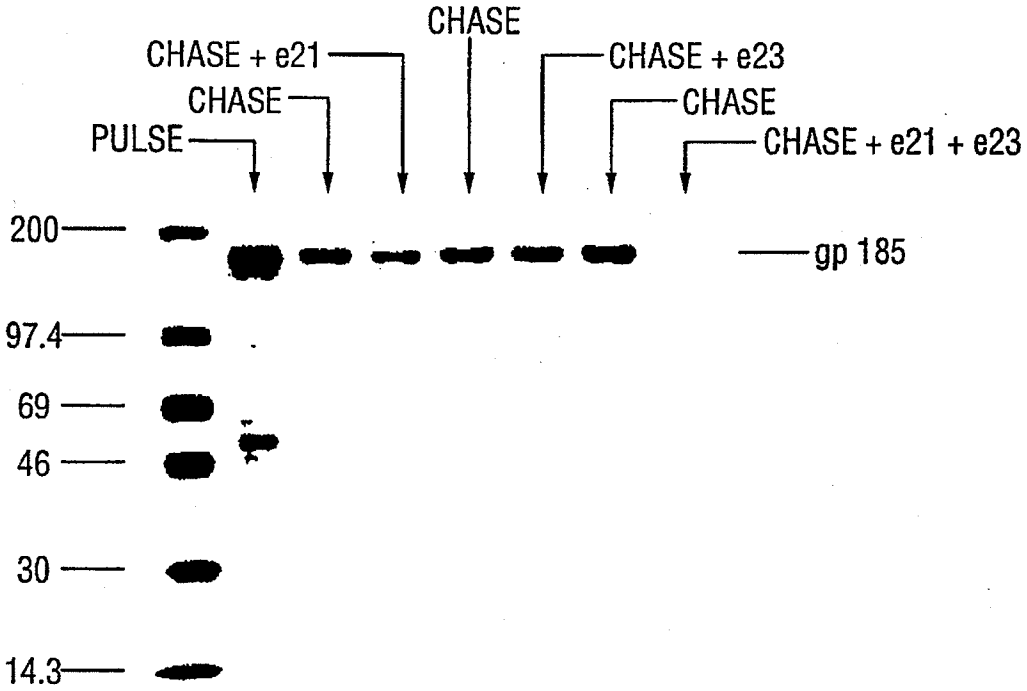
CHASE
CHASE + e21
CHASE + e23
CHASE
CHASE
PULSE
CHASE + e21 + e23
200
gp 185
97.4
69
46
30
14.3

FIG. 4B

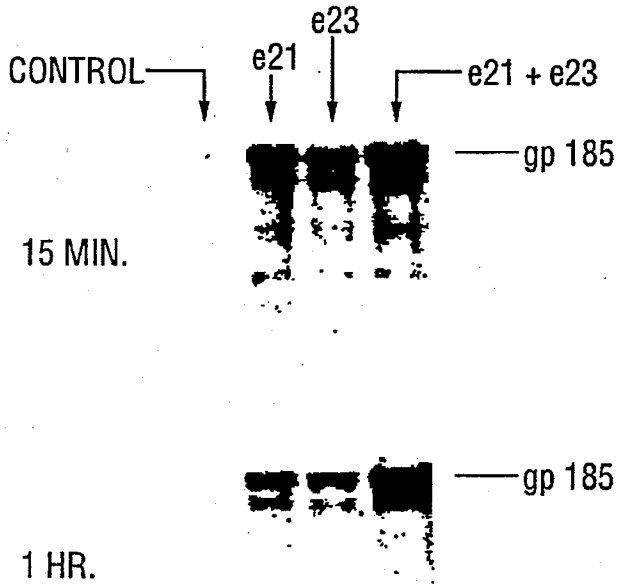
e23
e21
CONTROL
e21 + e23
gp 185
15 MIN.
gp 185
1 HR.

FIG. 5

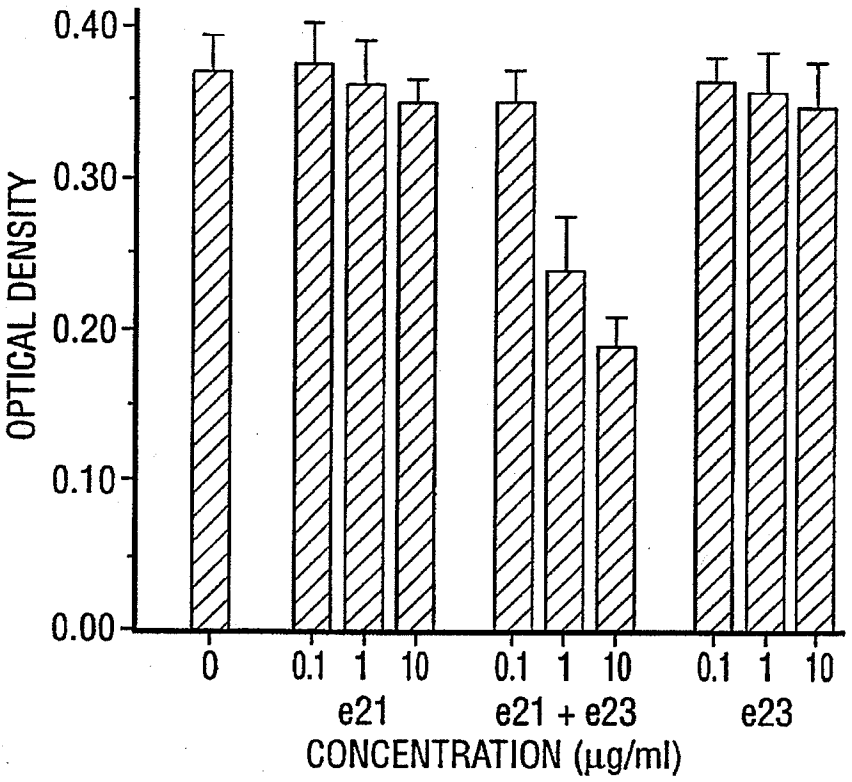
0.40
0.30
0.20
0.10
0.00
OPTICAL DENSITY
0
0.1
1
10
0.1
1
10
0.1
1
10
e21
e21 + e23
e23
CONCENTRATION (µg/ml)

FIG. 6

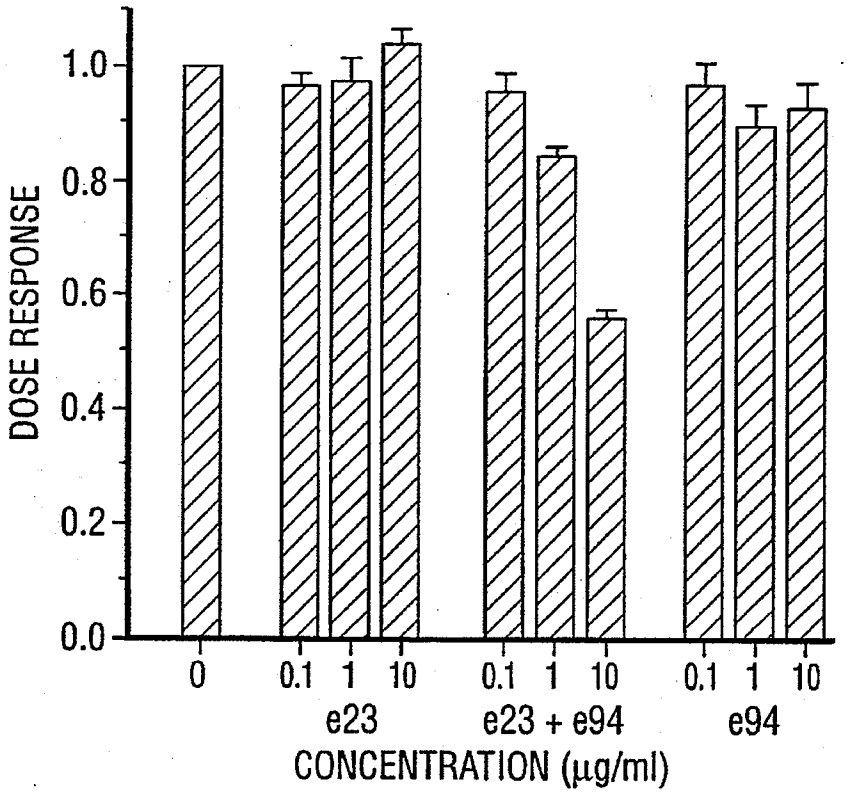
1.0
0.8
0.6
0.4
0.2
0.0
DOSE RESPONSE
0
0.1
1
10
0.1
1
10
0.1
1
10
e23
e23 + e94
e94
CONCENTRATION (µg/ml)

FIG. 7

```
ATGGACCTGCAGCTGACCCAGTCTCCAGCAATCCTGTCTGCATCTCCAGG
MetAspLeuGlnLeuThrGlnSerProAlaIleLeuSerAlaSerProGly

GGAGAAGGTCACAATGACTTGCAGGGCCACCCCAAGTGTAAGTTACATGC
 GluLysValThrMetThrCysArgAlaThrProSerValSerTyrMetHis

ACTGGTATCAGCAGAAGCCAGGATCCTCCCCCAAACCTTGGATTTATACC
  TrpTyrGlnGlnLysProGlySerSerProLysProTrpIleTyrThr

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ThrSerAsnLeuAlaSerGlyValProAlaArgPheSerGlyGlyGlySer

TGGGACCTCTTACTCTCTCACAGTCAGCAGAGTGGAGGCTGAAGATGCTG
 GlyThrSerTyrSerLeuThrValSerArgValGluAlaGluAspAlaAla

CCACTTATTACTGCCAGCAGTGGAGTCGTAGCCCACCCACGTTCGGAGGG
  ThrTyrTyrCysGlnGlnTrpSerArgSerProProThrPheGlyGly

GGGTCCAAGCTGGAAATAAAAGGTTCTACCTCTGGTTCTGGTAAATCTTC
GlySerLysLeuGluIleLysGlySerThrSerGlySerGlyLysSerSer

TGAAGGTAAAGGTGTGCAGCTGCAGGAGTCAGGACCTGAGGTGGTGAAGC
 GluGlyLysGlyValGlnLeuGlnGluSerGlyProGluValValLysPro

CTGGAGGTTCAATGAAGATATCCTGCAAGACTTCTGGTTACTCATTCACT
  GlyGlySerMetLysIleSerCysLysThrSerGlyTyrSerPheThr

GGCCACACCATGAACTGGGTGAAGCAGAGCCATGGAAAGAACCTTGAGTG
GlyHisThrMetAsnTrpValLysGlnSerHisGlyLysAsnLeuGluTrp

GATTGGACTTATTAATCCTTACAATGGTGATACTAACTACAACCAGAAGT
 IleGlyLeuIleAsnProTyrAsnGlyAspThrAsnTyrAsnGlnLysPhe

TCAAGGGCAAGGCCACATTTACTGTAGACAAGTCGTCCAGCACAGCCTAC
  LysGlyLysAlaThrPheThrValAspLysSerSerSerThrAlaTyr

ATGGAGCTCCTCAGTCTGACATCTGAGGACTCTGCAGTCTATTACTGTGC
MetGluLeuLeuSerLeuThrSerGluAspSerAlaValTyrTyrCysAla

AAGGAGGGTTACGGACTGGTACTTCGATGTCTGGGGCGCAGGGACCACGG
 ArgArgValThrAspTrpTyrPheAspValTrpGlyAlaGlyThrThrVal

TCACCGTCTCC
  ThrValSer
```

FIG. 8

```
ATGCAGCTGACCCAGTCTCCAGCAATCATGTCTGCATCTCCAGGGGAAAA  50
MetGlnLeuThrGlnSerProAlaIleMetSerAlaSerProGlyGluLys

GGTCACCATGACCTGCAGTGCCAGCTCAAGTGTAAGTAACATGCACTGGT  100
 ValThrMetThrCysSerAlaSerSerSerValSerAsnMetHisTrpTyr

ATCAGCAGAAGTCAAGCACCTCCCCCAAACTCTGGGTTTATGACACATCC  150
  GlnGlnLysSerSerThrSerProLysLeuTrpValTyrAspThrSer

AAACTGGCTTCTGGAGTCCCAGGTCGCTTCAGTGGCAGTGGGTCTGGAAA  200
LysLeuAlaSerGlyValProGlyArgPheSerGlySerGlySerGlyAsn

CTCTTACTCTCTCACGATCAGCAGCATGGAGGCTGAAGATGCTGCCACTT  250
 SerTyrSerLeuThrIleSerSerMetGluAlaGluAspAlaAlaThrTyr

ATTATTGTTATCAGGGGAGTGGGTACCCATTCACGTTCGGCTCGGGGACA  300
  TyrCysTyrGlnGlySerGlyTyrProPheThrPheGlySerGlyThr

AAGTTGGAAATAAAAGGTTCTACCTCCGGATCTGGTAAATCTTCTGAAGG  350
LysLeuGluIleLysGlySerThrSerGlySerGlyLysSerSerGluGly

TAAAGGTGTGCAGCTGCAGCAGTCTGGGGGTTGAGCTTGTCCGAGGAGGGG  400
 LysGlyValGlnLeuGlnGlnSerGlyValGluLeuValArgGlyGlyAla

CCTTAGTCAAGTTGTCCTGCAAAGCTTCTGACTTCAACATTAAAGACTAT  450
  LeuValLysLeuSerCysLysAlaSerAspPheAsnIleLysAspTyr

TATATCCACTGGGTGAAGCAGAGGCCTGAACAGGGCCTGGAATGGATTGG  500
TyrIleHisTrpValLysGlnArgProGluGlnGlyLeuGluTrpIleGly

ATGGATTCATCCTGAGAATGGTAATACTGTATATGACCCGAAATTCCAGG  550
 TrpIleHisProGluAsnGlyAsnThrValTyrAspProLysPheGlnGly

GCAAGGCCAGTATAACAGCAGACACATCCTCCAACGCGGCCTACCTTCAG  600
  LysAlaSerIleThrAlaAspThrSerSerAsnAlaAlaTyrLeuGln

CTCAGCAGCCTGACATCTGAGGACACTGCCGTCTATTACTGTGCTTCTTA  650
LeuSerSerLeuThrSerGluAspThrAlaValTyrTyrCysAlaSerTyr

TTACTACTATAGTGCTTACTATGCTATGTACTACTGGGGTCAAGGAACCT  700
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CGGTCACCGTCTCCTCATAA  720
  ValThrValSerSerTer
```

FIG. 9

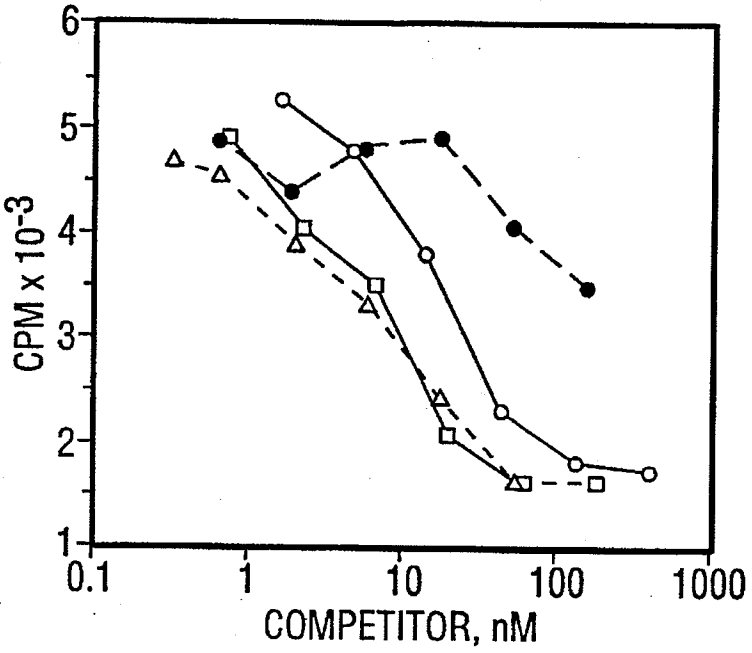

CPM x 10-3
6
5
4
3
2
1
0.1
1
10
100
1000
COMPETITOR, nM

FIG. 10

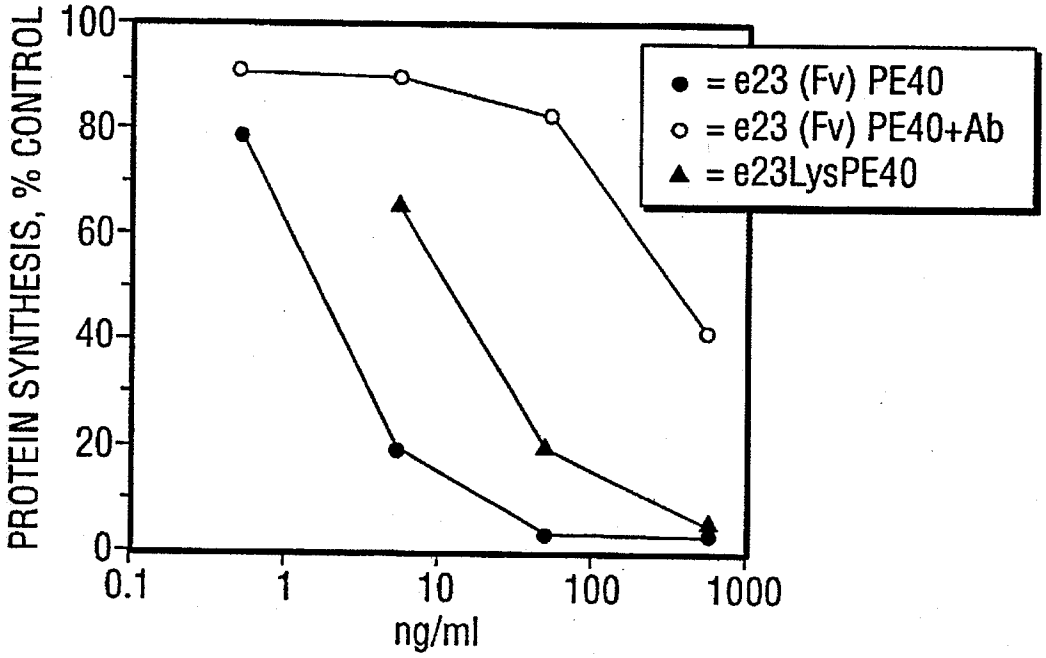

PROTEIN SYNTHESIS, % CONTROL
100
80
60
40
20
0
0.1
1
10
100
1000
ng/ml
● = e23 (Fv) PE40
○ = e23 (Fv) PE40+Ab
▲ = e23LysPE40

FIG. 11

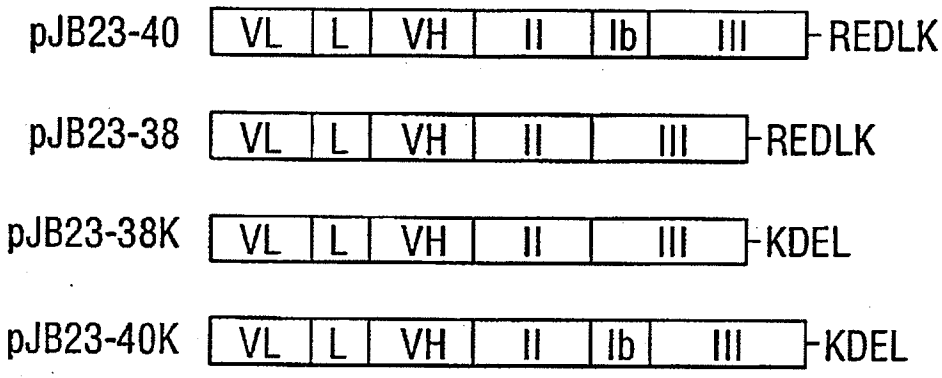

pJB23-40
VL
L
VH
II
Ib
III
REDLK
pJB23-38
VL
L
VH
II
III
REDLK
pJB23-38K
VL
L
VH
II
III
KDEL
pJB23-40K
VL
L
VH
II
Ib
III
KDEL

FIG. 12

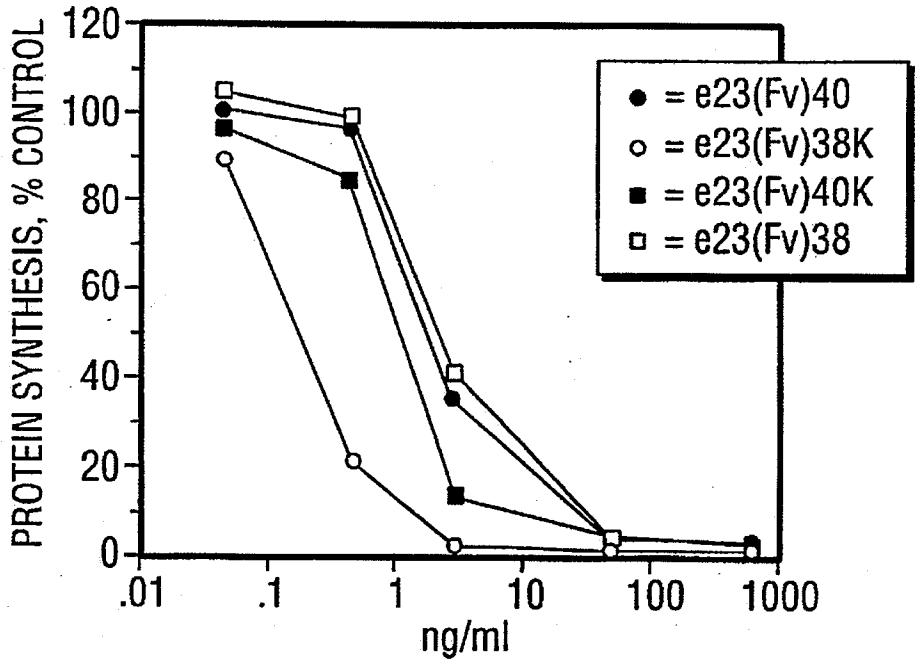
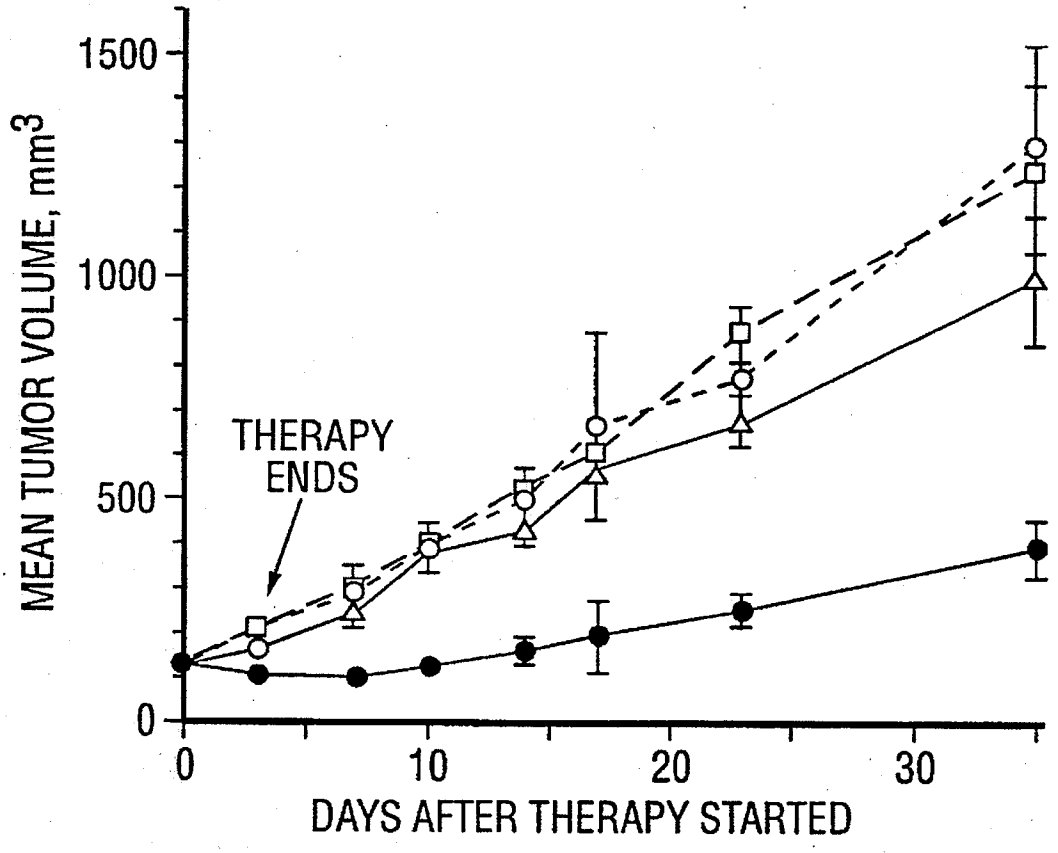

PROTEIN SYNTHESIS, % CONTROL
120
100
80
60
40
20
0
.01
.1
1
10
100
1000
ng/ml
● = e23(Fv)40
○ = e23(Fv)38K
■ = e23(Fv)40K
□ = e23(Fv)38

FIG. 13


1500
1000
500
0
MEAN TUMOR VOLUME, mm3
THERAPY ENDS
0
10
20
30
DAYS AFTER THERAPY STARTED

ANTI-ERBB-2 ANTIBODIES, COMBINATIONS THEREOF, AND THERAPEUTIC AND DIAGNOSTIC USES THEREOF

The subject application is a continuation-in-part of U.S. Pat. Ser. No. 07/906,555, pending, filed on Jun. 30, 1992, which is itself a continuation-in-part of U.S. Pat. Ser. No. 07/772,270, filed on Oct. 7, 1991, abandoned. These applications are incorporated by reference in their entirety herein.

BACKGROUND OF THE INVENTION

This invention relates to novel antibodies capable of specifically binding to the erbB-2, gp185$^{erbB\ 2}$ gene product, and combinations thereof.

The invention further relates to the use of antibodies capable of binding to the erbB-2, and/or combinations thereof, for treating and/or preventing the formation of erbB-2 expressing tumors.

The invention also relates to the use of antibodies capable of binding to the erbB-2 gene product, and/or combinations thereof, for the immunological detection of erbB-2 expressing tumor cells in an analyte.

The invention further relates to conjugates comprising novel antibodies capable of binding to gp185^{erbB-2}, and combinations thereof, wherein the antibody is bound to a cytotoxic moiety or a label which provides for the detection thereof, e.g., a fluorescent, enzymatic or radiolabel.

In particular, the invention relates to the use of monoclonal antibodies capable of specifically binding to gp185^{erbB-2}, and/or combinations thereof, and/or single chain antibodies derived therefrom for the treatment and/or prevention of erbB-2 expressing tumors. Additionally, the invention relates to the use of specific monoclonal antibodies or single chain antibodies capable of specifically binding to gp185^{erbB-2} for the detection of erbB-2 expressing tumor cells.

In recent years, evidence has been accumulated that growth factors and their receptors may be involved in the process of malignant transformation. The erbB-2 gene product (also called HER2, neu or c-erbB-2) encodes a 185-kDa growth factor receptor which has been implicated in the malignancy of some human adenocarcinomas. Specifically, the erbB-2 protein, gp185^{erbB-2}, is a receptor tyrosine kinase (Yarden et al, *Ann. Rev. Biochem.*, (1988), 57, 443–478) which is homologous to the epidermal growth factor (EGF) receptor. (Coussens et al, *Science*, (1985), 230, 1132–1139; Yamamoto et al., *Nature*, (1986), 319, 230–234.) The rat homologue of the gene undergoes oncogenic activation through a single point mutation. (Bargmann et al, *Cell*, (1986), 45, 649–657.)

The erbB-2 protein, like other receptor proteins, is composed of extracellular, transmembrane and intracellular domains. The extracellular domain contains two cysteine-rich areas and is 44% homologous to the epidermal growth factor receptor (EGFR). The intracellular domain contains a tyrosine kinase which is 82% homologous to that of EGFR. Because of these similarities to EGFR and to other tyrosine kinase receptors, it has been suggested in the literature that the c-erbB-2 protein may function as a growth factor receptor.

Clinical and experimental evidence suggests a role for overexpression of the erbB-2 protein in the progression of human breast, ovarian, and non-small lung carcinoma. For example, amplification and/or overexpression of the erbB-2 gene have been shown in 20–30% of breast adenocarcinomas (King et al, *Science*, (1985), 229, 974–976; Slamon et al, *Science*, (1987), 235, 177–182; Slamon et al, *Science*, (1989), 244, 707–712; Yokota et al, *Lancet*, (1986), 1,765–767; King et al, *Cancer Res.*, (1985), 49, 4185–4191), ovary adenocarcinomas (Slamon et al, *Science*, (1989), 244, 707–712), lung adenocarcinomas (Schneider et al, *Cancer Res.*, (1989), 49, 4968–4971) and stomach adenocarcinomas (Park et al, *Cancer Res.*, (1989), 49, 6605–6609). In breast carcinoma, a correlation has been observed between gene amplification and overexpression of erbB-2 protein and the aggressiveness of the malignancy (Slamon et al, Science, (1987), 237, 177–182; Slamon et al, *Science*, (1989), 244, 707–712). In cases of gene amplification, there is a resulting 50- to 100-fold increase in erbB-2 in RNA compared with normal cell levels (Kraus et al, *EMBO J.*, (1987), 6, 605–610). The overexpression of erbB-2 has also been directly linked to the malignant conversion of cancer cells. (DiFiore et al, *Science*, (1986), 237, 178–182; Hudziak et al, *Proc. Nat'l. Acad. Sci.*, (1987), 89, 7159–7163).

At least two lines of evidence suggest that erbB-2 overexpression may be involved in the pathogenesis of human neoplasia. First, as discussed supra, overexpression has been linked with poor prognosis in breast cancer, as well as ovarian cancer (Slamon et al, *Science*, (1989), 244, 707–712; Berchuk et al, *Cancer Res.*, (1990), 50, 4087–4091), stomach cancer (Yonemura et al, *Cancer Res.*, (1991), 51, 1004–1032) and lung cancer (Kern et al, *Cancer Res.*, (1990), 50, 5184–5191). Second, artificial overexpression of erbB-2 induces a transformed phenotype in NIH 3T3 fibroblasts (DiFiore et al, *Science*, (1986), 237, 178–182; Hudziak et al, *Proc. Nat'l. Acad. Sci.*, (1987), 84, 7159–7163), as well as in mammary epithelial cells (Pierce et al, *Oncogene*, (1991), 6, 1189–1194), suggesting that overexpression can not contribute directly to the development of the malignant phenotype.

Because of the extensive homology between the erbB-2 protein and the EGFR, it is widely assumed that the activation of growth signal transduction may proceed through similar mechanisms. One proposed mechanism involves receptor dimerization or oligomerization, which is thought to be an important step in the activation of EGFR intrinsic tyrosine kinase function (Yarden et al, *Biochemistry*, (1988), 27, 3114–3118; Schlessinger, *Biochemistry*, (1988), 27, 3119–3123). Interfering with receptor-receptor interactions has been evaluated as a potential therapeutic approach for the treatment of cancers associated with erbB-2 overexpression. In particular, such studies have involved the use of single monoclonal antibodies directed against the erbB-2 protein (Hudziak et al, *Mol. Cell. Biol.*, (1989), 9, 1165–1172) and the related EGFR protein (Divgi et al, *J. Nat'l. Cancer Inst.*, (1991), 83, 97–104) as potential therapeutic agents for the treatment of cancer.

The potential use of monoclonal antibodies for diagnosis and treatment of cancer has been studied extensively (Mellstadt, *Curr. Opinion Immunol.*, (1990), 2, 708–713). Receptors for growth factors constitute a desirable target for this approach because their location on the cell membrane renders them accessible to antibody molecules. Moreover, antibodies directed to growth factor receptors can potentially block biological functions essential for cell proliferation.

Previous studies have demonstrated in animal systems the potential therapeutic effects of monoclonal antibodies against the EGFR (Matsui et al, *Cancer Res.*, (1984), 44, 1002–1007; Aboud-Pirak et al, *J. Nat'l. Cancer Inst.*, (1988), 80, 1605–1611). Also, different monoclonal antibodies to the erbB-2 receptor have been found to inhibit the

proliferation of a human breast carcinoma cell line in human tissue culture (Hudziak et al, *Mol. Cell. Biol.*, (1989), 9, 1165–1172), and an antibody directed to the rat erbB-2 protein, has been reported to inhibit the tumorigenicity of fibroblasts transformed by the mutant rat erbB-2 oncogene (Drebin et al, *Proc. Nat'l. Acad. Sci.*, (1986), 83, 9126–9133; Drebin et al, *Oncogene*, (1988), 2, 387–399). Additionally, monoclonal antibodies which bind to erbB-2 have been used to study the biological function of the presumed receptor (McKenzie et al, *Oncogene*, (1989), 4, 543–548); Van Leenwen et al, *Oncogene*, (1990), 5,497,503; Fendly et al, *Cancer Res.* (1990), 50, 1550–1558).

While some monoclonal antibodies to the erbB-2 protein have shown promise as potential anticancer therapeutic agents, variable effects are observed dependent upon the particular monoclonal antibody. For example, Stancovski et al studied the in vivo effects of monoclonal antibodies on erbB-2 expressing tumors. Although some of the administered antibodies almost completely inhibited the growth in athymic mice of transfected murine fibroblasts that overexpress the erbB-2 protein, other antibodies were found to accelerate tumor growth or to result in intermediate responses (Stancovski et al, *Proc. Nat'l. Acad. Sci.*, (1991), 88, 8691–8695). Based on the variable observed effects, Stancovski et al postulated that anti-erbB-2 antitumor antibodies may affect both receptor functioning and host-tumor interactions.

Recently, the construction and bacterial expression of a bifunctional single chain antibody-phosphatase fusion protein targeted to the human erbB-2 receptor was reported by Weis et al, *Biotechnology*, (1992), 10, 1128–1132. Wels et al, reported that this fusion protein exhibits sufficient binding affinity to cells expressing the erbB-2 receptor to permit the use thereof as an immunohistochemical reagent for the detection of erbB-2 antigen on cells or tissues.

SUMMARY OF THE INVENTION

One object of the invention is to provide novel antibodies, preferably monoclonal or single chain antibodies derived therefrom, capable of specifically binding to erbB-2 protein, $gp185^{erbB-2}$, and which do not substantially bind to normal human cells which may be utilized for the treatment or prevention of erbB-2 expressing tumor cells, or for the immunological detection of erbB-2 expressing tumor cells.

Another object of the present invention is to provide a novel combination of antibodies comprising at least two different antibodies, preferably monoclonal or single chain antibodies which are capable of specifically binding to an extracellular domain of the erbB-2 protein, which antibodies are separately capable of treating and/or preventing the growth of human tumors associated by the overexpression of erbB-2, and wherein the combination results in greater cytotoxic activity than expected for the sum of the individual antibodies at the same overall antibody concentration.

Still another object of the invention is to provide novel therapeutic or diagnostic conjugates comprising anti-erbB-2 antibodies which are attached to a cytotoxic or detectable moiety.

Another object of the invention is to provide anti-tumor pharmaceutical compositions comprising an anti-tumor effective amount of the novel anti-erbB-2 specific antibodies or combinations thereof.

Another object of the invention is to provide immunodiagnostic compositions which comprise the novel anti-erbB-2 antibodies of the invention, preferably in combination with a moiety which provides for the detection thereof.

Still another object of the invention is to provide methods for the treatment and/or prevention of erbB-2 receptor overexpressing tumors comprising the administration of an anti-tumor effective amount of at least one of the disclosed anti-erbB-2 specific antibodies, or combination of erbB-2 specific antibodies. Preferably, such combinations of erbB-2 antibodies will exhibit better cytotoxic activity than would be expected for the sum of the cytotoxic activity of the individual antibodies at the same overall antibody concentration. Additionally, one or more of the administered antibodies may be conjugated to a cytotoxic moiety, e.g., an anti-tumor drug, toxin, or radionuclide.

Yet another object of the invention is to provide methods for the use of the disclosed erbB-2 binding antibodies and conjugates thereof for the immunological detection of erbB-2 expressing tumor cells.

DETAILED DESCRIPTION OF THE DRAWINGS

FIG. 1A. Specificity of monoclonal antibodies e21 and e23. Subconfluent SK-Br-3 monolayers were metabolically labeled with 355S-Cys (spec. act. 1000 Ci/mmol). Total cell proteins were immunoprecipitated with 10 μg of the indicated antibodies. The immune complexes were recovered by Protein G Agarose (Genex, Gaithersburg, Md.) and analyzed by SDS-PAGE on an 8–16% Tris-Glycine gel. The gel was exposed to film at –70° C. overnight with an intensifying screen.

FIG. 1B. $gp185^{erbB-2}$ overexpression in the gastric cell line N87 and a tumor from N87 mouse xenografts compared to high and low $gp185^{erbB-2}$ overexpressers. Cells or tumor were lysed in sample buffer which contained 0.125 M Tris-HCl, 4% SDS, 0.002% bromophenol blue, and 15% glycerol. 5% β-mercaptoethanol was added after the protein concentration was determined. Samples (10 μg total protein) were boiled for 3 min, fractionated by SDS-PAGE on 8–16% Tris-Glycine gel and transferred to nitrocellulose. Detection of $gp185^{erbB-2}$ was performed with a monoclonal antibody to the c-terminal portion of the protein.

FIG. 1C. Southern blot analysis of the erbB-2 gene in N87 (gastric), SK-Br-3 (breast), and SK-OV-3 (ovarian) cell lines and human placenta. DNA was extracted from cell lines and human placenta tissue using guanidine thiocyanate and cesium gradient centrifugation. DNA (15 μg) was cleaved with restriction enzyme HindIII, separated by electrophoresis on a 1% agarose gel, transferred to nitrocellulose, and probed with radioactive erbB-2 cDNA probe as previously described in King et al, *Cancer Res.* (1989), 49, 4185. The cDNA probe corresponds to the entire erbB-2 protein coding region.

FIG. 2. Effects of e21 and e23 on the growth of human N87 transferrin (10 μg/ml), 17-β-estradiol (10 nM), sodium selenite (5 nM), and 10 mM Hepes. PBS, e21, e23 or a combination of e21 and e23 at the indicated concentration were then added. The plates were grown at 37° C. in a 5% CO_2 humidified atmosphere. After 7 days, 50 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (0.1 mg) were added and allowed to incubate for 4 hours at 37° C. 90% of the media was then removed and the crystals solubilized in 0.175 ml DMSO. Optical densities were measured at 540 nm in a Molecular Devices Vmax kinetic microplate reader. Results are the average of eight wells with standard deviations noted. Under the conditions used, the cell number is directly proportional to MTT reduction.

FIG. 3A. Effects of treatment with e21, e23, a combination of e21 and e23, or PBS on the growth of N87 tumor xenografts in BNX mice. Tumor cells (5×10^6/mouse) were subcutaneously injected into the flanks of BNX (beige, nude, xid) mice. Treatment begun on day 1 consisted of four trial groups (3 mice per group) each given 0.2 ml intraperitoneal injections twice a week of either PBS, 200 μg purified e21 (0), 200 μg purified e23, or a mixture of 100/μg purified e21 and 100 μg of purified e23 for three weeks. Tumor growth is reported as an average relative tumor volume, s.e.m. ±15%. Two repeats of the experiment gave the same results.

FIG. 3B. Effect of treatment after the formation of small tumors. Cells were injected using the same treatment protocol as above except for the fact the treatment was begun 4 days after cell injection instead of 1 day after. Animal care was in accordance with institutional guidelines.

FIG. 4A. Effect of antibody binding on erbB-2 protein turnover. Subconfluent N87 cell monolayers were pulse-labeled 1 h with 20 μCi ^{35}S-Cysteine and then chased with 5 mM Cys in the presence of e21 alone, e23 alone, or a 1:1 combination of e21 and e23 (10 μg/ml) for 24 h. Total cellular protein was immunoprecipitated as described in FIG. 1 using a monoclonal antibody directed against the c-terminus of gp185^{erbB-2} coupled to Sepharose and analyzed by SDS-PAGE. The gel was exposed to film at –70° C. overnight with an intensifying screen.

FIG. 4B. Measurement of tyrosine phosphorylation of gp185^{erbB-2} after incubation with antibody combination. Cells were plated as described in the Materials and Methods infra. After 1 h cells were processed as described infra. Proteins were then electroblotter onto nitrocellulose paper and incubated with anti-phosphotyrosine IgG (monoclonal; Upstate Biotechnology, Inc.) and immunodetected using ^{125}I-protein A. The gel was exposed to film at –70° C. overnight with an intersifying screen.

FIG. 5. Effects of e21 and e23 on the growth of human Calu-3 lung adenocarcinoma tumor cells in a monolayer MTT growth assay. A single cell suspension of 10,000 cells/well was plated in a chemically defined medium consisting of RPMI-1640 supplemented with insulin (5 μg/ml), human transferrin (10 μg/ml), 17-β-estradiol (10 nM), sodium selenite (5 nM, and 10 mM Hepes. PBS, e21, e23 or a combination of e21 and e23 at the indicated concentration were then added. The plates were grown at 37° C. in a 5% CO_2 humidified atmosphere. After 7 days, 50 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (0.1 mg) were added and allowed to incubate for 4 hours at 37° C. 90% of the media was then removed and the crystals solubilized in 0.175 ml DMSO. Optical densities were measured at 540 nm in a Molecular Devices Vmax kinetic microplate reader. Results are the average of eight wells with standard deviations noted. Under the conditions used, the cell number is directly proportional to MTT reduction.

FIG. 6. Effects of e23 and e94 on the growth of human Calu-3 lung adenocarcinoma tumor cells in a monolayer MTT growth assay. A single cell suspension of 10,000 cells/well was plated in a chemically defined medium consisting of RPMI-1640 supplemented with insulin (5 μg/ml), human transferrin (10 μg/ml), 17-β-estradiol (10 nM), sodium selenite (5 nM), and 10 mM Hepes. PBS, e23, e94 or a combination of e21 and e23 at the indicated concentration were then added. The plates were grown at 37° C. in a 5% CO_2 humidified atmosphere. After 7 days, 50 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (0.1 mg) were added and allowed to incubate for 4 hours at 37° C. 90% of the media was then removed and the crystals solubilized in 0.175 ml DMSO. Optical densities were measured at 540 nm in a Molecular Devices Vmax kinetic microplate reader. Results are the average of eight wells with standard deviations noted. Under the conditions used, the cell number is directly proportional to MTT reduction.

FIG. 7. [SEQ ID NO. 1] The cDNA sequence for the *single chain anti-erbB-2 antibody, containing heavy and light variable domains derived from e23 (e23(Fv)).

FIG. 8. [SEQ ID NO. 2] The cDNA sequence for the *single chain anti-erbB-2 antibody, containing heavy and light variable domains derived from e21 (e21(Fv)).

FIG. 9. Depicts the binding of SC(Fv) and immunotoxin to erbB-2. The ability of purified e23(Fv)(O) and e23(Fv)PE38KDEL (●) to inhibit the binding of I^{125}-labeled e23 Fab was measured using cells overexpressing erbB-2 N87 as the binding target. Also shown are e23 Fab (□) and intact antibody e23 (Δ).

FIG. 10. Depicts the cytotoxicity of e23(Fv)Pe40 on BT474 cells as shown by inhibition of protein synthesis. Results are shown as percentage of control cells to which no toxin was added, ●, e23(Fv) PE40 alone; o, e23(Fv)PE40 plus e23 (20 μg/ml); ▲, e23-LysPE40.

FIG. 11. Depicts schematically the e23(Fv)PE40 derivatives which were synthesized. VH, variable region of heavy chain; VL, variable region of light chain; L, linker; II, domain II of PE; Ib, domain Ib of PE; III, domain III of PE. Carboxyl-terminal amino acid sequences are shown in single letter code.

FIG. 12. Depicts the comparative cytotoxic activities of various e23(Fv)PE40 derivatives on BT474 cells, ●, e23(Fv)PE40; o, e23(Fv)PE38KDEL; e23(Fv)PE40KDEL; □, e23 (Fv)PE38.

FIG. 13. Depicts results of therapy of tumors formed in mice by human gastric cell line N87. Tumors were established by injection of 5×10^6 N87 cells subcutaneously on the backs of BNX mice. Therapy was initiated 7 days following injection of cells. Treatments were twice daily injections in the tail vein with 2 μg of e23(Fv)PE38KDEL(●), LysPE38KDEL(o), or e23Fab(Δ) or with phosphate-buffered saline (□). Measurements were conducted externally with calipers.

DETAILED DESCRIPTION OF THE INVENTION

One object of the invention is to provide novel antibodies capable of specifically binding to the erbB-2 protein, and do not substantially bind to human cells which antibodies may be used for the treatment and/or prevention of erbB-2 expressing tumors, or the detection of erbB-2 expressing tumor cells. In particular, the invention is directed to novel murine monoclonal antibodies capable of specifically binding to erbB-2 protein, gp185^{erbB-2}, and single chain antibodies containing variable heavy and light domains derived therefrom. More particularly, the invention is directed to novel monoclonal antibodies designated e23, e21 and e94, and single chain antibodies containing variable heavy and light domains derived therefrom, the synthesis of which is set forth infra.[1]

[1]The e21, e23 and e94 antibodies disclosed herein are respectively identical to Ab#21, Ab#23 and Ab#94 disclosed in the parent applications 07/906,555 and 07/772,270. The nomenclature has been changed so that it is consistent with current literature relations to these antibodies.

Another object of the invention is to provide novel combinations of at least two different antibodies capable of

binding to the erbB-2 receptor, wherein the antibodies are separately capable of treating and/or preventing the expression of erbB-2 expressing tumor cells. Preferably, these antibody combinations will exhibit greater (synergistic) cytotoxic activity than would be expected for the sum of the individual antibodies if administered separately at the same overall antibody concentration. The present inventors have unexpectedly discovered that the combination of at least two different anti-erbB-2 antibodies may provide for greater cytotoxicity than would be expected for the same overall concentration of each individual antibody if administered separately.

Preferably, at least two of these antibodies will recognize distinct erbB-2 epitopes and will not cross react with the other. However, the present inventors do not want to be restricted thereby, but rather intend to embrace any combination of anti-erbB-2 antibodies, which provides for synergistic cytotoxic activity. In the preferred embodiment, the antibody combinations will comprise e23 and e21 or e23 and e94.

The erbB-2 antibodies which are suitable for use in such combinations may include monoclonal antibodies capable of binding to erbB-2, recombinant erbB-2 antibodies, chimeric erbB-2 antibodies which comprise constant and variable domains derived from different species, such as murine-human chimeric antibodies (wherein the constant domain is human and the variable domain is of murine origin), humanized forms of such antibodies, single chain antibodies capable of binding to the erbB-2 protein, and fragments or analogues of erbB-2 monoclonal antibodies which are capable of binding to the erbB-2 protein, and which antibody combinations exhibit synergistic cytotoxic activity. Additionally, the invention further embraces the use of bispecific antibodies, in particular bispecific antibodies comprising the fusion of two different erbB-2 binding single chain antibodies, preferably each of such single chain antibodies binding to a distinct erbB-2 epitope.

Such erbB-2 antibodies will be made by conventional methods. For example, erbB-2 monoclonal antibodies may be obtained through genetic recombination or via Kohler-Milstein fusion techniques. Methods for making antibodies by recombinant methods are well known in the art and include, e.g., the methods disclosed by Cabilly et al, U.S. Pat. No. 4,816,567 and Boss et al, U.S. Pat. No. 4,816,347.

Hybridoma cell lines which secrete e23 and e21 were both deposited on Apr. 4, 1994 at the American Type Tissue Collection in Rockville, Md. and been respectively designated ATCC Accession Nos. HB11601 and HB11602. Both of these cell lines were deposited in accordance with the Budapest Treaty. All restrictions as to the availability of these cell lines will be irrevocably removed upon issuance of a patent in this application, or any other application which claims benefit to this application or to which this application claims benefit.

Methods for making single chain antibodies having binding specificity to a predetermined antigen are also known and are disclosed, e.g., in U.S. Pat. No. 4,946,778 to Ladner et al, U.S. Pat. No. 4,704,692 to Ladner, and U.S. Pat. No. 4,939,666 to Hardman. Methods for making single chain antibody fusions are disclosed in Chaudhary et al, *Proc. Nat'l. Acad, Sci.*, (1990), 87, 1066–1070.

As discussed supra, the claimed antibody combinations will provide for the treatment and/or prevention of cancers associated by the overexpression of erbB-2 protein. Preferably, the first and second antibodies will be combined such that the resultant ratio of the first to second antibody is effective for decreasing the expression of the erbB-2 protein, since the expression of this protein is implicated in the prognosis of some cancer types. More preferably, the combination of antibodies will provide for enhanced decrease of erbB-2 protein expression relative to the same concentration of the individual antibodies contained therein. A convenient method for assaying the expression of the erbB-2 gene product comprises studying the effect of antibody binding on erbB-2 protein turnover (the results of which are depicted in FIG. 4). However, any method which provides for the quantitation of erbB-2 expression, e.g., Northern analysis, should be applicable. The decrease in the expression of the erbB-2 gene product is believed to result from the administered antibody or antibodies decreasing the half-life of the erbB-2 protein in the cell.

Antibody combinations which exhibit synergistic activity will be selected on the basis of those antibodies which in combination reduce erbB-2 receptor expression greater than any of the individual antibodies at the same overall antibody concentration. The relative ratios of the respective antibodies which are contained in the subject antibody combinations will vary dependent upon the number of different antibodies, their epitopic specificity, isotype, binding affinity, etc. An example of a suitable ratio of the first and second antibodies (assuming that the combination comprises two different erbB-2 antibodies) comprises a ratio of from about 1:2 to about 2:1. Preferably, the ratio will be about 1:1.

However, the invention is not restricted to any particular antibody ratio, but rather embraces any combination of at least two different erbB-2 specific antibodies which provide for reduced erbB-2 receptor expression, and preferably provides for a synergistic reduction in erbB-2 receptor expression and inhibition of erbB-2 expressing tumor cells as a result of the selected combination of erbB-2 antibodies.

As discussed supra, another object of the invention is to provide novel methods and compositions for the treatment and/or prevention of erbB-2 expressing tumor cells comprising the administration of a therapeutically effective amount of at least one of the subject novel erbB-2 monoclonal antibodies or single chain antibodies of the invention, namely e21, e23, e94 (the preparation of which is disclosed in the following examples), single chain antibodies containing variable heavy and light domains derived therein, and antibodies having the identifying characteristics thereof. Identifying characteristics are intended to include those properties which affect the immuno-binding of the particular anti-erbB-2 antibody, in particular epitopic specificity.

These antibodies may be administered by themselves, or these antibodies may be attached to cytotoxic moieties, which include e.g., radioactive materials, anti-cancer drugs (e.g., daunomyocin, adriamycin, chlorambucil), anti-metabolites (e.g., methotrexate), inhibitors of protein synthesis (e.g., diptheria toxin, Pseudomonas exotoxin, ricin, abrin, etc.), and agents which bind DNA (such as alkylating agents). In a preferred embodiment, the subject erbB-2 specific antibodies will be attached to a Pseudomonas exotoxin A variant which lacks the N-terminal domain (domain I) which is responsible for the binding of the exotoxin to its corresponding cell receptor. A particularly preferred Pseudomonas exotoxin A variant is PE40, the sequence of which is disclosed e.g., in Hwang et al, *Cell*, (1987), 48, 129–136 and Siegall et al, *J. Biol. Chem.*, (1989), 264, 14256–14261 which references are incorporated by reference herein. Thereby, the subject anti-erbB-2 antibody Pseudomonas A exotoxin conjugates only comprise the binding function of the erbB-2 antibodies, and do not exhibit non-specific binding attributable to the exotoxin. In a more preferred embodiment, the Pseudomonas exotoxin A vari-

ants which are attached to the subject erbB-2 antibodies will also comprise a deletion of amino acids 365–380 (which eliminates a disulfide bond) and/or the deletion of the five C-terminal amino acids and the substitution of a tetrapeptide, KDEL, therefor. Such Pseudomonas exotoxin A variants are known in the literature, and are described e.g., in Brinkmann et al, *Proc. Nat'l. Acad. Sci.*, (1991), 88, 8616–8620; Hwang et al, *Cell*, (1987), 48, 129–136; Seetharam et al, *J. Biol. Chem*, (1991), 266, 17376–17381; and Siegall et al, *J. Biol. Chem.*, (1989), 264, 14256–14261, which references are incorporated by reference in their entirety.

Preferably, the antibody which is administered will comprise e23, its corresponding single chain antibodies, humanized antibodies derived from e23, chimeric antibodies, (containing the variable sequences therefrom and human constant domain sequences) or therapeutic diagnostic conjugates thereof since this antibody has been found to bind to erbB-2 expressing tumor cells with high affinity, and to not substantially bind to normal human tissues. This is advantageous both for diagnostic and therapeutic applications since it avoids false positives (if the antibodies are utilized diagnostically to assay for cancers associated with overexpression of $gp185^{erbB\text{-}2}$) and also avoids cytotoxicity to normal human cells when the antibodies are used therapeutically since this antibody does not substantially bind to normal human cells.

Avidin-biotin immunoperoxidase binding assays with e23 and a number of different normal tissues have established that e23 only reacts with the membrane of simple, stratified, and squamous epithelial cells in normal human tissues. (These assays and the results thereof are described infra). This narrow range of reactivity with normal human cells is unexpected given that the erbB-2 receptor is known to be comprised on a large number of normal human tissues. It is known e.g., that erbB-2 is expressed throughout the intestinal, respiratory, urinary and reproductive tracts, as well as the skin of fetal and adult specimens (Press et al, *Oncogene*, (1990), 5, 953–962). More particularly, the erbB-2 receptor is expressed in normal uterine endometrial tissues (Brumm et al, *Virhous Archiv. A Pathol. Anat.*, (1990), 417,477–484; normal breast tissues (Tsutsumi et al, *Hum Pathol*, (1990), 21, 750–758; Potter et al, *Histopathology*, (1989), 15,351–362) and kidney tissues (Potter et al, *Int. J. Cancer*, (1989), 44,969–974).

It has been suggested in the literature that the erbB-2 receptor may be expressed in a different form on the surface of cancer tissues than normal cells. Accordingly, the low reactivity observed with e23 and normal human tissues may be attributable to it binding an erbB-2 epitope which is selectively expressed on erbB-2 receptor expressing tumor cells.

The subject antibodies or antibody combinations will be administered to cancer patients having cancers associated by overexpression of erbB-2 protein by standard routes of administration, e.g., intraperitoneally via intravenous, or intramuscular routes. The effective dosage of the antibodies will vary dependent e.g., upon the particular antibody or antibody combination which is administered, whether or not the antibody or antibodies are conjugated to a therapeutic effective moiety, the particular therapeutic moiety (e.g., a toxin, radioactive moiety, or alkylating agent), and the condition of the patient being treated.

Preferably, the effective concentration at the target tumor site will be at least 1 μg/ml, and will not exceed 10 μg/ml. In general, in order to achieve the desired concentration of the antibody or antibodies at the tumor site the antibody or antibody combination will be administered at a dose ranging from about 0.1 mg/kg to about 10 mg/kg of body weight.

As noted supra, the subject anti-erbB-2 antibodies may be administered in unmodified form, or one or more of the subject antibodies may be administered on the form of immunotoxins. Methods for the construction of immunotoxins are well known in the art. Generally, such techniques involve direct covalent attachment or the complexation of an antibody to a therapeutic moiety, e.g., an anti-cancer pharmaceutical agent, a cytotoxin, a radioactive compound (e.g., isotopes of boron and rhenium); agents which bind DNA, such as alkylating agents, anti-cancer compounds (e.g., daunomycin, adriamycin, chlorambucil); anti-metabolites (e.g., methotrexate); and inhibitors of protein synthesis (e.g., diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas auerogi-nosa*, ricin A chain, abrin A chain, modaccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolacca americana* proteins (PAPI, PAPII, and PAP-S), Momordica charantia inhibitor, curcin, crotin, Sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocoin, phenomycin and enomycin. Alternatively, the subject antibodies may be attached to a desired therapeutic-moiety using known bifunctional protein coupling agents. Examples of such coupling reagents are SPDP, IT, bifunctional derivatives of imidoesters such as dimethyl adipimidate HCl, active esters such as disuccinimidyl suberate, glutaraldehyde, bis-azido compounds, bis-diazonium compounds and diisocyanates of bis-active fluorine compounds. Preferably, the attachment of the antibody to a therapeutic moiety will be effected in a manner which minimizes loss of antibody binding function.

If the administered erbB-2 antibody or antibodies comprises a single chain antibody, the antibody will preferably be attached to a therapeutic moiety since such antibodies lack the Fc effector portion of the antibody. In such cases, attachment will be effected by expressing a DNA fusion of the subject single chain erbB-2 antibody coding sequences and a DNA sequence which encodes for a polypeptide exhibiting therapeutic activity. Generally, such therapeutic moieties will comprise polypeptide toxins or active variants thereof. The construction of such single chain antibody-toxin fusions is exemplified infra in the examples. Moreover, methods for expressing single chain antibody-therapeutic moiety DNA fusions are disclosed in U.S. Pat. No. 5,132, 405 by Huston et al. In the preferred embodiment the subject anti-erbB-2 single chain antibodies will be fused to variants of the Pseudomonas exotoxin A which lack the N-terminal binding domain, and which may be further modified to eliminate disulfide bond formation (e.g., by deletion of amino acids 365–380) and/or which comprise deletions of the five C-terminal amino acids and the substitution of a tetrapeptide, KDEL, therefor. Such Pseudomonas exotoxin A variants are known in the literature as discussed supra.

The administration of the subject anti-erbB-2 antibodies in combination with a discrete therapeutic moiety, e.g., a cis-platinum type compound is further envisioned since it has been reported that a monoclonal antibody directed to erbB-2 enhances the cytotoxicity of cis-diamminedichloroplatinum against human breast and ovarian tumor cell lines. (Hancock et al, *Cancer Res.*, (1991), 51, 4575–4580).

For parenteral administration the subject antibodies or antibody combinations may be formulated in a unit dosage injectable form (solution, suspension, emulsion) in association with a pharmaceutically acceptable parenteral vehicle. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin.

Nonaqueous vehicles such as mixed oils and ethyl oleate may also be used. Liposomes may also be used as carriers. The vehicles may contain minor amounts of additives that enhance isotonicity and chemical stability, e.g., buffers and preservatives. The subject antibodies and antibody combinations will typically be formulated in such vehicles at concentrations of about 1 μg/1 ml to 10 μgl/ml.

As noted, a preferred embodiment of the invention will comprise administration of a therapeutically effective amount of a combination of at least two different anti-erbB-2 antibodies. The present inventors have found that combinations of anti-erbB-2 antibodies may provide for synergistic cytotoxic activity. In the most preferred embodiments, the combination of antibodies will comprise e21 and e23, or e23 and e94. However, the invention is not restricted to such combinations, but rather embraces any combination of erbB-2 antibodies which provides for enhanced cytotoxic activity then the equivalent overall concentration of the individual antibodies contained in the combination.

Preferably, the different antibodies will bind to different erbB-2 epitopes and will not cross-react with each other. Although the present inventors do not want to be limited to any theoretical reasoning, a possible mechanism by which the down regulation and protection and/or killing of human cancer cells overexpressing erbB-2 is achieved by the present invention is that the antibodies act by constraining the erbB-2 gene product, $gp185^{erbB-2}$ into an activated conformation thus mimicking an agonist ligand.

The results disclosed herein demonstrate that a combination of erbB-2 receptor binding antibodies leads to different and more potent anti-tumor activities than single antibodies. More specifically, the results indicate that combination antibody therapy is a useful strategy for treatment of malignancies associated by overexpression of 185^{erbB-2}. This approach may be particularly important in the treatment of cancers such as gastric cancer which respond poorly to currently available chemotherapies.

As discussed supra, another object of the invention relates to the use of the novel erbB-2 monoclonal and single chain antibodies of the invention as diagnostic agents for assaying for the presence of $gp185^{erbB-2}$ overexpressing tumor cells in analytes, e.g., blood serum and tumor biopsies.

The use of anti-erbB-2 specific monoclonal antibodies as diagnostic agents for detecting erbB-2 cancer cells is known in the art, as disclosed e.g. in U.S. Pat. No. 4,938,948 to Ring et al., and WO 89/06692 by Hudziak et al, published on Jul. 27, 1989. The $gp185^{erbB-2}$ binding antibodies of the invention should be particularly suitable as diagnostic agents given their high binding affinity to the erbB-2 receptor. It is especially envisioned that e23 and single chain antibodies derived therefrom should be advantageous diagnostic agents, given the narrow range of binding of this antibody to normal human tissues. Thus, the use of e23 and its corresponding single chain antibody should reduce the likelihood of false positive binding results. This is a particular concern with assays which measure erbB-2 receptor expression since this protein is known to be comprised in detectable amounts on a large number of different normal human tissues.

Essentially, a sample suspected of containing erbB-2 expressing cancer cells will be incubated with the subject antibody or antibodies for a sufficient time to permit immune reactions to occur. Those skilled in the art will recognize that there are many variations in these basic procedures. These variations include, for example, RIA, ELISA, precipitation, agglutination, complement fixation and immunofluorescence. Preferably, the subject antibodies will be labelled to permit the detection of antibody-erbB-2 immunocomplexes.

The labels that are used in making labeled versions of the antibodies include moieties that may be detected directly, such as radiolabels and fluorochromes, as well as moieties, such as enzymes, that must be reacted or derivatized to be detected. The radiolabel can be detected by any of the currently available counting procedures. The preferred isotope labels are ^{99}Tc, ^{14}C, ^{131}I, ^{125}I, ^{3}H, ^{32}P and ^{35}S. The enzyme label can be detected by any of the currently utilized calorimetric, spectrophotometric, fluorospectrophotometric or gasometric techniques. The enzyme is combined with the antibody with bridging molecules such as carbodiimides, periodate, diisocyanates, glutaraldehyde and the like. Many enzymes which can be used in these procedures are known and can be utilized. Examples are perioxidase, alkaline phosphatase, β-glucuronidase, β-D-glucosidase, urease, glucose oxidase plus peroxidase, galactose oxidase plus peroxidase and acid phosphatase. Fluorescent materials which may be used include, for example, fluorescein and its derivatives, rhodamine and its derivatives, auramine, dansyl, umbelliferone, luciferia, 2,3-dihydrophthalazinediones, horseradish peroxidase, alkaline phosphatase, lysozyme, and glucose-6-phosphate dehydrogenase. The antibodies may be tagged with such labels by known methods. For instance, coupling agents such as aldehydes, carbodiimides, dimaleimide, imidates, succinimides, bis-diazotized benzadine and the like may be used to tag the antibodies with the above-described fluorescent, chemiluminescent, and enzyme labels. Various labeling techniques are described in Morrison, *Methods in Enzymology*, (1974), 32B, 103; Syvanen et al., *J. Biol. Chem.*, (1973), 284, 3762; and Bolton and Hunter, *Biochem J.*, (1973), 133, 529.

The antibodies and labeled antibodies may be used in a variety of immunoimaging or immunoassay procedures to detect the presence of cancer in a patient or monitor the status of such cancer in a patient already diagnosed to have it. When used to monitor the status of a cancer, a quantitative immunoassay procedure must be used. If such monitoring assays are carried out periodically and the results compared, a determination may be made regarding whether the patient's tumor burden has increased or decreased. Common assay techniques that may be used include direct and indirect assays. If the sample includes cancer cells, the labeled antibody will bind to those cells. After washing the tissue or cells to remove unbound labeled antibody, the tissue sample is read for the presence of labeled immune complexes. In indirect assays the tissue or cell sample is incubated with unlabeled monoclonal antibody. The sample is then treated with a labeled antibody against the monoclonal antibody (e.g., a labeled antimurine antibody), washed, and read for the presence of ternary complexes.

For diagnostic use the antibodies will typically be distributed in kit form. These kits will typically comprise: the antibody in labeled or unlabeled form in suitable containers, reagents for the incubations for an indirect assay, and substrates or derivatizing agents depending on the nature of the label. $gp185^{erbB-2}$ controls and instructions may also be included.

The following examples are offered to more fully illustrate the invention, but are not to be construed as limiting the scope thereof.

The following materials and methods were used in Examples 1–5.

MATERIALS AND METHODS

Monoclonal Antibodies. Mice were immunized using a membrane preparation of N/erbB-2 cells (NIH/3T3 cells

engineered to overexpress the human erbB-2 protein). Following tests of polyclonal antibody response using immunoprecipitation, three fusions were conducted using the myeloma cells line Ag8.653 and standard techniques. These fusions produced approximately 1500 hybridoma clones which were each screened using enzyme-linked immunosorbent assay. Membranes isolated from N/erbB-2 cells were bound to polystyrene plates, and culture medium was added to allow antibody-antigen interaction. Immunoglobulin binding was detected using a biotinylated goat antimouse antibody, streptavidin horseradish peroxidase, and o-phenylenediamine hydrochloride. Positive reacting hybridomas were picked and counter-screened using membranes from wild-type NIH/3T3 membranes. The molecular specificity was confirmed by immunoprecipitation analysis. Five hybridomas were picked with anti-erbB- 2-specific reactivity and cloned by limiting dilution; three of these were designated as e21, e23 and e94. Ascites was prepared by administering injections of 10^6 hybridoma cells to pristane-primed mice. Antibodies were isolated in large amounts from ascites fluid and purified by high-performance liquid chromatography with a Gammabind Ultra column (Genex, Gaithersburg, Md.). SDS-PAGE[2] was run under nonreducing conditions using Coomassie blue staining with a single band at M_r 180,000 observed, indicating a >98% purified preparation. From 1 ml of ascites approximately 8–15 mg of antibody were routinely purified.

[2]The abbreviations used are" SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; MTT, 3-(4–5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; cDNA, complementary DNA.

Cells Lines and Tissue Culture.

The human gastric tumor cell line used in these studies, N87, has been previously described in the literature and was routinely subcultured in RPMI 1640 supplemented with 10% fetal bovine serum. The cell lines SK-Br-3, MDA-MB-468, and MDA-MB-231 (breast) and SK-OV-3 (ovarian) were routinely subcultured in improved minimal essential medium (IMEM) supplemented with 5% fetal bovine serum. Cultures were maintained in humidified incubators at 37° C. in an atmosphere of 5% CO_2 and 95% air. Cells were tested for Mycoplasma using a ribosomal RNA hybridization method (GenProbe, San Diego, Calif.).

Growth Inhibition Assays.

A single cell suspension of 10,000 cells/well was plated in a serum-free defined media of RPMI 1640 containing bovine serum insulin (5/μg/ml final concentration) was then added. The plates were incubated for 5–7 days in a CO_2 incubator with humidity at 37° C. Cell viability was monitored by one of two different methods. The first, the MTT assay (Mossman et al, *J, Immun. Meth.*, (1983), 65, 55–63), is based on the ability of live cells to reduce a tetrazolium-based compound, MTT, to a purplish colored formazan product that can be measured spectrophotometrically. After 7 days, 50 μl of MTT reagent (0.1 mg) were added and allowed to incubate for 4 h at 37° C. Ninety % of the media was then removed, and the crystals were solubilized in 0.175 ml dimethyl sulfoxide with absorbance measured at 540 nm in a Molecular Devices Vmax kinetic microplate reader. The second method involves the cell number measurement in monolayer cultures by crystal violet staining (Sibler et al, *Anal. Biochem.*, (1989), 182, 16–18). Cells were plated as above and after 7 days cells were fixed by the addition of 20 μl of a 11% glutaraldehyde solution. After being shaken on a Bellco Orbital Shaker for 15 min the plates were washed three times with deionized water. Plates were then air-dried and stained by the addition of 100 μl of a 0.1% solution of crystal violet dissolved in 200 mM borate, pH 6.0. After being shaken for 20 min at room temperature, excess dye was removed by extensive washing with deionized water, and the plates were air-dried prior to solubilization in 100 μl of 10% acetic acid. Absorbance was measured at 590 nm in the microplate reader.

Antibody Specificity.

Subconfluent SK-Br-3 monolayers were metabolically labeled with [^{35}S]Cys (specific activity, 1000 Ci/mmol). Total cell proteins were immunoprecipitated with 10 μl of the indicated antibodies. The immune complexes were recovered by protein G-Agarose (Genex) and analyzed by SDS-PAGE on an 8–16% Tris-glycine gel. The gel was exposed to film at –70° C. overnight with an intensifying screen.

Western Blots.

Cells or tumors were lysed in sample buffer which contained 0.125 M Tris-HCl, 4% SDS, 0.002% bromophenol blue, and 15% glycerol. Five % α-mercaptoethanol was added after the protein concentration was determined. Samples (10 μg total protein) were boiled for 3 min, fractionated by SDS-PAGE on 8–16% Tris-Glycine gel (Novex, Encinitas, Calif.), and transferred to nitrocellulose. Detection of gp185^{erbB-2} was performed with a monoclonal antibody (E2-4001; Molecular Oncology, Inc. to the COOH-terminal portion of the protein.

Southern Blots.

DNA was extracted from cell lines and human placenta tissue using guanidine thiocyanate and cesium gradient centrifugation. DNA (15 μg) was cleaved with restriction enzyme HindIII, separated by electrophoresis on a 1% agarose gel, transferred to nitrocellulose, and probed with a radioactive erbB-2 cDNA prove as previously described (King et al, *Cancer Res.*, (1989), 49, 4185–4191). The cDNA probe corresponds to the entire erbB-2 protein coding region.

In Vivo Antitumor Assay.

Tumor cells (5×10^6/mouse) were injected s.c. into the flanks of BNX (beige, nude, xid) mice. The day after cell inoculation treatment was begun which consisted of four trial groups (3 mice/group), each given 0.2-ml i.p. treatment injections twice a week. Tumor growth was monitored at least once a week and reported as an average relative tumor volume. The effect of treatment after the formation of small tumors was also carried out. Cells were injected using the same treatment protocol as above except that the treatment was begun 4 days after cell injection instead of 1 day after. Animal care was in accordance with institutional guidelines. Statistical analysis was carried out using a SAS Computer Package (SAS Institute, Cary, N.C.).

gp185^{erbB-2} Stability Assay.

Subconfluent N87 cell monolayers were pulse-labeled 1 h with 20 μCi[^{35}S]cysteine and then chased with 5 mM Cys in the presence of antibody for 24 h. Total cellular protein was immunoprecipitated as described above using a monoclonal antibody directed against the COOH terminus of gp185^{erbB-2} coupled to Sepharose and analyzed by SDS-PAGE. The gel was exposed to film at –70° C. overnight with an intensifying screen.

Tyrosine Phosphorylation.

Cells were plated as in the protein stability assay. After 1 h, cells were processed, and proteins were extracted in sample buffer for electrophoresis as the antibody specificity experiment. Following electrophoresis in the proteins were electroblotted onto nitrocellulose paper and incubated with anti-phosphyotyrosine IgG (monoclonal; Upstate Biotechnology, Inc.) and immunodetected using ^{125}I-protein A. The gel was exposed to film at –70° C. overnight with an intensifying screen.

EXAMPLE 1

Preparation of Antibodies and Immunological Characterization Thereof

The procedure described in the Materials and Methods Supra was used to produce three monoclonal antibodies which were designated e21; e23; and e94.

In particular, monoclonal antibodies directed against the extracellular domain of gp185^{erbB-2} were tested for specific reaction to N/erbB-2 cell membranes in an ELISA assay. Two of these designated e21 and e23 after screening in growth assays exhibited the highest biological activity and were subjected to further testing.

Both antibodies specifically immunoprecipitated a single ^{35}S-labeled protein of MW 185,000 from SK-Br-3 cells, a breast cancer cell line which overexpresses gp185^{erbB-2} protein, (Kraus et al, *EMBO J.*, (1987), 6, 605) as shown in FIG. 1A. No immunoprecipitation was detected in cells which do not overexpress the gp185^{erbB-2} protein (e.g., MDA-MB-468, data not shown).

Because the erbB-2 oncogene is overexpressed frequently in at least 20% of stomach cancer, which have a poor clinical course, the effect of these antibodies on cell proliferation was studied on a gastric cell line, N87, which overexpresses gp185^{erbB-2} at levels commensurate with SK-Br-3. An immunoblot of the N87 cell line and a nude mouse tumor xenograft from N87 is shown in FIG. 1B compared to the breast cell lines SK-Br-3 (high level of gp185^{erbB-2} overexpression) and MDA-MB-231 (low level of gp185^{erbB-2} overexpression). The levels of erbB-2 gene amplification in N87 as shown in FIG. 1C surpassed those found in the well characterized SK-Br-3 and SK-OV-3 cell lines (Kraus et al, *EMBO J.*, (1987), 6, 605).

EXAMPLE 2

Effect of Antibodies on Tumor Growth In Vitro

A dose response analysis of the effects of the antibodies on N87 cell proliferation is shown in FIG. 2. Antibodies e21 or e23 administered individually had no effect on the monolayer growth of cells up to a concentration of 10/μg /ml (6 μM). Administration of a 1:1 combination of e21 and e23, however, markedly affected cell proliferation at doses as low as 1 μg/ml. Fab fragments prepared from both antibodies also had no effect on cell growth alone or in combination (data not shown). In analogous experiments with three other gastric cell lines displaying little or no overexpression by immunoblot analysis, no inhibition of growth even at the highest dose was observed with the antibody combination or the antibodies alone.

EXAMPLE 3

Preventive Combination Antibody Therapy

The efficacy of combination antibody therapy was tested on the growth of N87 tumor xenografts. One inoculation of five million N87 cells were injected subcutaneously into nude mice produced rapidly growing tumors with a short latency. Tumor growth at the injection site was easily quantitated. As shown in FIG. 3A, the N87 cells did not form tumors in the animals treated twice a week for three weeks with a total of 200 μg of antibodies per injection with the combination of e21 and e23. In sharp contrast they were potently tumorigenic in animals treated with the single antibodies or PBS and the tumor grew to over 1 cm^3 in tumor volume over the period measured. In contrast to in vitro experiments, each monoclonal antibody alone may have limited activity to partially restrict the rate of tumor growth. However, the activity exhibited by the combination far exceeded the cumulative effect expected from the combination.

To determine if the combined therapy with e21 and e23 was able to eradicate established tumors, an experiment was performed in which tumors were allowed to grow to measurable sizes prior to antibody treatment. The results are illustrated in FIG. 3B. In animal groups randomized so that the starting size of the tumors was near the same volume (100 mm^3), the tumors continued to grow when the animals were given single antibody treatment of e21 or e23 (200 μg/injection, 2 injection/week, 3 weeks, 6 mice). In contrast, in the animals given two antibody combination treatment of e21 and e23, results shown are the average of 6 animals, tumors completely regressed after 11 days (4 treatments of 200 μg of total antibody). This is the first reported observation of tumor xenograft regression induced by a combination of different anti-erbB-2 monoclonal antibodies. Previous studies have shown that two anti-neu antibodies can inhibit the growth of tumors by murine cells transformed by the mutationally activated neu oncogene (Drebin et al, *Oncogene*, (1988), 2, 273). The activation of the murine neu oncogene is accomplished by point mutation as evidenced by qualitative interference in the structure and function of the neu gene, whereas the human erbB-2 oncogene is activated by overexpression of erbB-2, a quantitative interference of the apparently normal protein which results in tumor formation.

Since the mechanisms for tumor growth are so different between murine and human, it was unexpected that similar mechanisms of neutralization of the genes involved would be effective. This effect is also seen with the inhibition of leukemic tumor cell growth using anti-transferrin monoclonal antibodies (White et al, *Cancer Res.*, (1990), 50, 6295).

EXAMPLE 4

Antiproliferative Effects of Antibody Combination

To investigate the molecular basis for the antiproliferative effects of e21 and e23, we measured the rate of gp185^{erbB-2} turnover in the presence or absence of antibodies. N87 cells were pulse-labeled with ^{35}S-Cys and then chased for various times in the presence of single antibody or the e21/e23 combination. The results of a 24 h chase are shown in FIG. 4A. The antibody gp185^{erbB-2} combination induced rapid degradation of gp185^{erbB-2} while the individual antibody treatment had little or no effect. Thus, the antiproliferative effect of e21/e23 treatment might likely be explained by their ability to increase the turnover of gp185^{erbB-2}.

EXAMPLE 5

Autophosphorylation Activity of Antibody Combination

To test whether the subject antibodies result in increased gp185^{erbB-2} autophosphorylation, anti-phosphyotyrosine immunoblots were effected using N87 cells. Cells were plated as described in the Materials and Methods. After 1 h, cells were processed, and proteins were extracted in sample buffer for electrophoresis when evaluating antibody specificity. Following electrophoresis the proteins were electrob-

lotted onto nitrocellulose paper and incubated with anti-phosphyotyrosine IgG (monoclonal; Upstate Biotechnology, Inc.) and immunodetected using ^{125}I-protein A. The gel was exposed to film at –70° C. overnight with an intensifying screen.

As shown in FIG. 4B, increases in the tyrosine phosphorylation of gp185^{erbB-2} from N87 cells were observed after 15 min and 1 h after the addition of the single antibodies or the antibodies in combination. (The same results were observed at 30 min and 2 h; data not shown). This suggests that activation of tyrosine activity may be necessary but is probably not essential for growth inhibition.

EXAMPLE 6

Combination Antibody Treatment Effect the Growth of Calu-3 Cells

In order to demonstrate that the effect of combination of anti-erbB-2 antibodies e21 and e23 is not limited to effect on the N87 gastric cancer cell line, we investigated the human lung adenocarcinoma cell line Calu-3. This cell line over-expresses the gp185^{erbB-2} protein as determined by immunoblot analysis (data not shown). In experiments very similar to that described above, the combination of e21 and e23 show dramatic inhibition of cell growth as measured in an MTT assay (FIG. **5**). In this experiment, a single cell suspension of 10,000 cells/well was plated in a chemically defined media consisting of RPMI-1640 supplemented with insulin, human transferrin, 17-estradiol. sodium selenite, and Hepes buffer. PBS, e21, e23 or a combination of e21 and e23 were then added. The cells were allowed to grow at 37° C. in a 5% CO_2 humidified atmosphere. After 7 days, MTT reagent was added and allowed to incubate for 4 hours at 37° C. 90% of the media was then removed and the crystals solubilized in DMSO. Optical densities were measured at 540 nm in a Molecular Devices Vmax kinetic microplate reader. A dose response analysis of the effects of antibody treatment is shown in FIG. **5**. These results indicate that combination antibody therapy is not limited in effectiveness to N87 cells or gastric cancer cells. It also indicates that combination antibody therapy may have effectiveness in the treatment of adenocarcinoma of the lung.

EXAMPLE 7

Effectiveness of other Combinations of Antibodies in Inhibiting Cell Growth

In order to determine if e21 and e23 are unique in their ability to combine to cause growth inhibition, we investigated the combination of e94 and e23 on the growth of Calu-3 cells in vitro. e94 is another monoclonal antibody developed by the present inventors which specifically binds to the erbB-2 protein. An MTT assay of cell growth was conducted as described in EXAMPLE 6. As shown in FIG. **6**, the combination of antibodies inhibits cell growth and the individual antibodies do not. This indicates that the ability to combine antibodies to produce a more profound growth inhibition is not limited to a particular antibody combination.

EXAMPLE 8

Generation of a single chain (Fv) from mAb e23

Poly A RNA was extracted from e23 producing hybridoma cells using oligo dT affinity chromatography (In vitrogen). cDNA was prepared using random primer (N_6) (Boerhinger Mannheim). The immunoglobulin light and heavy chain clones were isolated using PCR and the primers: light chain, [SEQ ID NO. 3] 5' CAC GTC GAC ATT CAG CTG ACC CAC TCT CCA and [SEQ ID NO. 4] GAT GGA TCC AGT TGG TGC AGC ATC3'; heavy chain [SEQ ID NO. 5] 5'C GGA ATT TCA GGT TCT GCA GIA GTC WGG3' and [SEQ ID NO. 6] 5' AGC GGA TCC AGG GGC CAG TGG ATA GAC3' [G,A,C,T stand for standard nucleotides; I for inosine, W for A or T]. The products of the PCR reaction were cloned into p^{uc18}. Linkage into a SC(Fv) was by PCR giving the individual light and heavy cDNA clones and 4 oligonucleotides [SEQ ID NOS. 7–10]

5'- cgagatgagtccagctgacccagtctc

5'- gaagatttaccagaaccagaggtagaaccttttatttccagcttgga

5'- ctggttctggtaaatcttctgaaggtaaaggtgtgcagctgcaggag

5'- cgagtgcaagcttaggagacggtgaccgt.

The light and heavy chain coding regions were joined by a synthetic linker GSTSGSGKSSEGKG specified by overlapping oligonucleotides as described. The intact scFv coding region was inserted in frame with an *E. coli* OMPA leader sequence under direction of the lambda P_L promoter. Induction of protein and bacterial lysis and refolding was as previously described (Pantaliano et al, *Biochem.*, (1991), 30, 117–125). scFv was purified as a single peak from CM chromatography and judged to be >70% by SDS gel electrophoresis. This scFv will be referred to herein as e23(Fv).

EXAMPLE 9

Generation of a scFv from mAb e21

Poly A RNA was extracted from e21 producing hybridoma cells using oligo dT affinity chromatography (In vitrogen). cDNA was prepared using random primer (N_6) (Boerhinger Mannheim). The immunoglobulin light and heavy chain clones were isolated using PCR and the primers: light chain, [SEQ ID NO. 3] 5' CAC GTC GAC ATT CAG CTG ACC CAC TCT CCA and [SEQ ID NO. 4] GAT GGA TCC AGT TGG TGC AGC ATC3'; heavy chain [SEQ ID NO. 5] 5'C GGA ATT TCA GGT TCT GCA GIA GTC WGG3' and [SEQ ID NO. 6] 5' AGC GGA TCC AGG GGC CAG TGG ATA GAC3'[G,A,C,T stand for standard nucleotides; I for inosine, W for A or T]. The products of the PCR reaction were cloned into pUC18. Linkage into a scFv was by PCR giving the individual light and heavy cDNA clones and 4 oligonucleotides [SEQ ID NOS. 7–10] 5'- cgagatgagtccagctgacccagtctc

5'- gaagatttaccagaaccagaggtagaaccttttatttccagcttgga

5'- ctggttctggtaaatcttctgaaggtaaaggtgtgcagctgcaggag

5'- cgagtgcaagcttaggagacggtgaccgt.

The light and heavy chain coding regions were joined by a synthetic linker [SEQ ID NO. 11] GSTSGSGKSSEGKG specified by overlapping oligonucleotides as described. The intact scFv coding region was inserted in frame with an *E. coli* OMPA leader sequence under direction of the lambda P_L promoter. Induction of protein and bacterial lysis and refolding was as previously described (Pantaliano et al, *Biochem.*, (1991), 30, 117–125). scFv was purified as a single peak from CM chromatography and judged to be >70% by SDS gel electrophoresis. This scFv will be referred to herein as e21(Fv).

EXAMPLE 10

Construction of Single-Chain Immunotoxin with Anti-erbB2 Antibody e23

Single-chain immunotoxins made by the fusion of the antigen-binding region (Fv) and PE40 can retain the binding affinity of the native antibody and are often more active than

the respective chemical conjugates (Chaudhary et al, *Proc. Nat'l. Acad. of Sci.*, (1990), 87, 1066–1070; Batra et al, *Mol. Cell Biol.* (1991), 11, 2200–2205.) For this reason, we selected antibody e23 for construction of a first-generation recombinant immunotoxin. The construction of the subject e23 antibody derived single chain antibody immunotoxins is also described in Butra et al, *Proc Natl Acad Sci,* (1992), Vol. 89, pp. 5867–5871, which is incorporated by reference in its entirety herein.

First, an intact sc(Fv) for coding region designated e23(Fv) was generated as described supra. The sequence of the single-chain protein is shown in (FIG. **7**). To verify the binding activity of the purified e23(Fv) protein we conducted competition binding using ^{125}I-labeled e23 Fab (see FIG. **9**). The overall structure of our first recombinant immunotoxin is the amino-terminal e23 sc(Fv) domain joined to the translocation (II) and ADP-ribosylating (III) domains of PE. The assembled gene is under control of a bacteriophage T7 promoter. The resulting plasmid, pJB23–40, expresses the variable region of the light chain of e23, a 14-amino acid linker peptide, the variable region of the heavy chain of e23, and amino acids 253–613 of PE. The chimeric protein was expressed in *E. coli* and purified. The resulting protein was >70% pure as judged by SDS/PAGE (data not shown).

Cytotoxicity of e23(Fv)PE40.

e23(Fv)PE40 was tested on BT474 breast cancer cells and was found to inhibit protein synthesis in a dose-dependent manner with an ID_{50} of 1.5 ng/ml (FIG. **10**, Table 1). The cytotoxic activity was blocked by competition with excess native e23, demonstrating the specificity of e23(Fv)PE40 for erbB-2-containing cells (FIG. **10**). Another anti-erbB-2 monoclonal antibody, e21, which binds to a different site, had no effect on the toxicity of e23(Fv)PE40 (data not shown). In the same experiment, e23-LysPE40, a chemical conjugate comprising e23 chemically conjugated to PE40, had an ID_{50} of 12 ng/ml (FIG. **10**, Table 1). The activity of e23(Fv)PE40 was assayed on several cell lines expressing erbB-2 and compared with that of the chemical conjugate, e23-Lys40 (Table 1). On all four target cells, e23(Fv)PE40 was active and the ID_{50} values on a molar basis were 2- to 3-fold lower than those of e23-LysPEA0. Both molecules had very little activity on KB cells, showing their specificity for erbB-2-expressing cells.

Derivatives of e23(Fv)PE40.

It is known that when the five carboxy terminal amino acids of the Pseudomonas exotoxin variant PE40 are replaced by KDEL that this results in molecules that have 3- to 10-fold higher cytotoxic activities See Maram et al, *J. Biol. Chem.*, (1991), 266, 17376–17381. Also, deleting part of domain Ib of PE, specifically amino acids 365–380, thereby deleting a disulfide bond, does not result in any loss of activity of several fusion proteins including TGFα-PE40, anti-Tac(Fv)-PE40, and B3(Fv)PE40, Brinkmann et al, *Proc. Nat'l. Acad. Sci.*, (1991), 88, 8616–8620; Siegall et al, *J. Biol. Chem.*, (1989), 264, 14256–14261 . Since mixed disulfide bonds can form during the renaturation of recombinant proteins, we thought it would be helpful to delete this region. To explore the possibility of making a much more active derivative of e23(Fv)PE40, we made three new constructions: (i) e23(Fv)PE40KDEL, where the five carboxyl terminal amino acids of PE40 are replaced by KDEL; (ii) e23(Fv)PE38, in which amino acids 365–380 have been deleted from e23(Fv)PE40 but the carboxyl terminus is not altered; and (iii) e23(Fv)PE38KDEL, where the five C-terminal amino acids are replaced by KDEL in e23(Fv)PE38. These derivatives are diagramed in FIG. **11**. The chimeric proteins were also purified to >70% purity and tested for cytotoxic activity on target cells. As shown in FIG. **12**, all of the new derivatives inhibited the protein synthesis of BT474 cells in a dose-dependent manner, with e23(Fv)PE38KDEL being the most active. Table 2 summarizes the ID_{50} values of the e23(Fv)PE40 derivatives on various cell lines. On all three target cell lines, e23(Fv)PE38KDEL, was found to be the most active. e23(Fv)PE38KDEL was 6- to 10-fold more active than e23(Fv)PE40 (Table 2). None of the proteins had any cytotoxicity on KB cells, a cell line that does not overexpress erbB-2. In the presence of excess e23, the cytotoxic activity of all derivatives was abolished (data not shown). The binding activity of e23(Fv)PE38KDEL was monitored in a competition binding assay. As shown in FIG. **9**, e23(Fv)PE38KDEL was able to compete with homologous e23 Fab for binding, but a higher concentration was required than for e23(Fv). This result is consistent with either a lower overall affinity of e23(Fv)PE38KDEL or the purified protein being a mixture of active and inactive species. Current purification methods for e23(Fv)PE38KDEL do not allow us to separate forms on the basis of binding activity. To verify the binding activity of the e23(Fv), we conducted a similar competition binding assay and found that e23 Fab binds with slightly lower affinity than intact antibody and monomeric Fab produced from e23 (FIG. **9**).

Growth Inhibition of Human Tumors in a Nude Mouse Model.

The selective toxicity of the e23(Fv)PE38KDEL to cells overexpressing erbB-2 encouraged us to attempt to treat human tumor cells growing in nude mice. The human gastric cancer cell line N87 has been shown to overexpress erbB-2 protein at high levels as a result of gene amplification, and N87 cells grow well as a subcutaneous tumor in immunocomprised mice Kaspyrzyk et al, *Cancer Res.*, (1992), 52, 2771–2776. Injections of 5×10^6 cells on day 0 were followed by six intravenous treatments over 3 days, starting on day 10. Immunotoxin treatment inhibited growth of established tumors (FIG. **13**). No animal deaths were observed at doses of 2 μg. Equivalent amounts of either e23 Fab, e23 SC(Fv) (data not shown), or LysPE38KDEL had no effect on tumor growth. Nonspecific toxicity was assayed by monitoring the animal weight; no weight loss was observed at doses of 2 μg.

TABLE 1

Comparison of activity of e23(Fv)PE40 and chemical conjugates on various human cells lines ID_{50} ng/ml (pM)

Cells	e23(Fv)PE40	e23-LysPE40	Relative activity*
BT474	1.5 (23)	12 (63)	0.37
N87	3.5 (54)	24 (126)	0.43
SK-OV-3	22.0 (338)	180 (947)	0.36
SK-Br-3	32.0 (492)	180 (947)	0.52
A431	170.0 (2615)	>500 (>2631)	NC

*Ratio of ID_{50}(pM) of e23(Fv)PE40 to ID_{50} of e23-LysPE40 on the same cell line.
NC, not calculated.

TABLE 2

Activity of e23(Fv)PE40 and derivatives on various human cell lines

Protein	BT474	N87	SK-OV-3	KB
e23(Fv)PE40	3	8	80	>500
e23(Fv)PE40KDEL	1.6	3.8	22	>500
e23(Fv)PE40KDEL	3.6	3.7	62	>500
e23(Fv)PE38KDEL	0.18	1.2	5	>500

Based upon the above results, it is expected that the subject erbB-2 immunotoxins, most particularly e23(Fv)PE38KDEL should be suitable for treatment of cancers associated by the overexpression of $gp185^{erbB-2}$ which occurs, e.g., in about 30% of adenocarcinomas of the breast, stomach, lung and ovary. Direct evidence supporting this conclusion is provided by the results showing inhibition of tumor growth in nude mice.

EXAMPLE 11

The following experiment analyzes the expression of the antigen recognized by murine monoclonal antibody e23 on a panel of normal human tissues, using cryostat-cut frozen tissue sections and the avidin-biotin immunoperoxidase technique. These studies were conducted at the highest concentration of antibody that does not show non-specific binding. This allows for the detection of all levels of cross-reactivity in different tissues. In addition, fixation analyses to establish the best combination of antigenic showing intensity and morphological preservation was performed.

MATERIAL AND METHODS

Source of the tissues

Histologically normal adult human tissues were obtained from surgical pathology and autopsy specimens. Fresh tissues were embedded in cryomolds of OCT compound (Miles Laboratories, Inc., Naperville, Ill.), frozen, and stored at −70° C. until needed. All tissues were used as frozen tissue sections from IMPATH's frozen tissue bank, and were well-preserved histologically.

Reagents

e23 was used at a concentration of 1.0 mg/ml, and was used diluted in phosphate buffered saline (PBS, NACl-8.54 gms and $NaPo_4$-1.46 gms per liter, pH=7.2–7.4) (DIFCO Laboratories, Detroit, Mich.) containing 2% bovine serum albumin (BSA). e23 was received on wet ice and stored at 4° C.

Purified mouse IgG1 (Coulter Immunology, Hialeah, Fla.), was used as the negative control, diluted in 2% BSA/PBS to the same working concentration as Antibody e23.

Biotinylated horse anti-mouse IgG (heavy + light chains specific) affinity purified antibodies (1:200 dilution in PBS) (Vector Laboratories, Inc., Burlingame, Calif.) were used as a secondary antibody.

Avidin-biotin-peroxidase complexes (1:50 dilution in PBS) (Vector Laboratories, Inc., Burlingame, Calif.) were used as the labeling reagent.

Immunohistochemistry

Immunoperoxidase Techniques: Immunohistochemical studies were performed using the Avidin-Biotin Immunoperoxidase technique. To assure that tissue sections adhere, slides were coated with 0.005% Poly-L-Lysine solution in deionized water (Sigma Chemical Co., St. Louis, Mo.). Frozen sections were cryostat-cut (4–8 microns thick), air dried and fixed in 95% ethanol, then bathed in PBS.

Endogenous peroxidase activity was blocked with a 10 minute 0.3% hydrogen peroxide incubation.

To reduce endogenous biotin content, all tissues were washed several times in PBS, then incubated with a solution of avidin (Vector Labs) for 15 minutes at room temperature, washed in PBS, incubated with a solution of biotin (Vector Labs) for 15 minutes at room temperature, and washed in PBS.

Tissue sections were then incubated for 10 minutes with suppressor serum. Suppressor serum consisted of normal horse serum (NHS) (Jackson ImmunoResearch Labs, West Grove, Pa.) diluted to 5% with PBS containing 2% BSA.

Tissue sections were incubated with the primary antibody for 30 minutes at room temperature. Sections were then washed, incubated with biotinylated horse anti-mouse IgG antibodies for 20 minutes, washed in PBS, incubated with avidin-biotin-peroxidase complexes for 20 minutes, then washed in PBS.

The peroxidase reaction was performed by incubating tissue sections for 2–5 minutes with 3,3-diaminobenzidine-tetrahydrochloride (DAB) (Sigma Chemical Co., St. Louis, Mo.). Tissue sections were thoroughly washed, counterstained with Harris hematoxylin, alehydrated through graded alcohols and xylenes, and mounted with permaslip (Alba Scientific, St. Louis, Mo.).

Tissues that demonstrated high levels of background staining with the negative control antibody were repeated, utilizing more extensive washing and avidin-biotin blocking.

Controls

erbB-2 positive human breast carcinoma (92-31-626) was used as positive control for Antibody e23; erbB-2 negative human breast carcinoma (92-31-625) was used as the negative control tissue for e23. Both control tissues were supplied by Oncologix, Inc. Negative controls consisted of substitution of the primary antibody with purified mouse IgG 1, used at the same working concentration as test antibody e23.

Fixation

The purpose of the fixation analysis is to establish the conditions which provide the optimal combination of antigenie staining intensity and morphological preservation. Frozen tissues used in our studies are fixed after they have been sectioned, and fixatives are only used in subsequent studies if the staining intensity is better than in unfixed sections. For this study, positive control tissue fixed with four fixative reagents were compared to unfixed tissue. These fixatives included 10% neutral buffered formalin, acetone (2°–8° C.), methyl/acetone (1:1 V/V; 2°–8° C.) and 95% ethanol. Fixation in 95% ethanol gave optimal results for e23.

Titration

The purpose of the titration analysis is to determine the highest concentration of the antibody that can be used for the study without non-specific binding. This allows for detection of all levels of cross-reactivity in different tissues. This analysis showed a concentration of 10.0/μg /ml to be optimal.

GLP

All aspects of the study were conducted in adherence to the FDA Good Laboratory Practices regulations.

The results of this analysis are set forth on the following pages.

RESULTS OF SPECIFICITY ANALYSIS
Antibody e23
Summary of Reactivity on Normal Tissues

	Tested Positive/Total	Range of Reactivity (0 to 3+)
Neg. Control Tissue-Breast Carcinoma	0	0
Pos. Control Tissue-Breast Carcinoma	90–100% of the tumor cells	3+
Adrenal:		
Cortex	0/3	0
Medulla	0/3	0
Bladder:	3/3	+/– to 3+
Bone Marrow:	0/3	0
Peripheral Blood Cells:	0/3	0
Brain:		
Neuroglia	0/1	0
Neurons	0/1	0
Brain Cerebellum:		
Neuroglia	0/4	0
Neurons	0/4	0
Brain Cortex:		
Neuroglia	0/2	0
Neurons	0/2	0
Breast:		
Acini	3/3	1+ to 3+
Ducts	3/3	1+ to 3+
Cervix:		
Endocervix	3/3	2+ to 3+
Exocervix	3/3	1 + F to 3+
Esophagus:		
Squamous epithelium	2/3	1+ to 3+
Glands	2/2[1]	3+
Eye:	0/3	0
Heart:	0/3	0
Kidney:		
Glomerulus	0/3	0
Tubules	0/3	0
Collecting Tubules	0/3	0
Large Intestine:	3/3	+/–
Liver:		
Hepatocytes	0/3	0
Bile ducts	0/3	0
Kupffer cells	0/3	0
Lung:		
Bronchial cells	3/3	2+
Alveolar cells	3/3	1+ to 2+
Lymph Node:	0/3	0
Muscle, skeletal:	0/3	0
Ovary:	0/3*	0
*Mesothelial cells reactive (2–3+) in 1 of 3 specimens; ovary negative.		
Pancreas:		
Endo. Cells	0/3	0
Exo. Cells	0/3	0
Ducts	0/3	0
Parathyroid:	0/3	0
Parotid Gland:	3/3	1+ to 2+
Pituitary:	0/3	0
Prostate:	3/3*	1+ to 2+
*Stromal cells (fibroblasts) are reactive (2–3+) in 3 of 3 specimens.		
Skin:		
Epidermis	3/3	1+
Adnexa	3/3	1+
Small intestine:	3/3	1+ to 3+
Spinal Cord:		
Neuropil	0/3	0
Neurons	0/3	0
Spleen:	0/3	0
Stomach:	3/3	1 + F
Skeletal muscle:	0/3	0
Thymus:	0/2	0
Testis:		
Germinal cells	0/3	0
Stromal cells	0/3	0
Thyroid:	0/3	0
Tonsil:	0/3	0
Uterus:		
Endometrium	2/2[2]	1+ to 2+
Myometrium	0/3	0
Stromal cells	0/3	0

[1]Glands not seen in 1 of 3 specimens.
[2]Endometrium not seen in 1 of 3 specimens.

Murine monoclonal antibody e23 was supplied by Oncologix, Inc. to IMPATH for analysis of immunoreactivity with a selected panel of normal human tissues. Antibody e23 is of the IgG 1 subclass and was supplied at a concentration of 1.0 mg/ml. Fixation analysis indicated that the optimal fixative for Antibody e23 was 95% ethanol. Using 95% ethanol as the fixative of choice, a titration analysis was run using the positive control tissue, human breast carcinoma (92-31-626), supplied by Oncologix, Inc. The purpose of the titration analysis is to determine the highest concentration of the antibody that can be used for the study without non-specific binding. This allows for detection of all, including low, levels of cross-reactivity of the antibody in different tissues. Since the components that give rise to non-specific binding are not tissue specific, the titration analysis is done on the positive control tissue. Titration analysis with e23 indicated that the optimal working concentration for the antibody was 10.0 μg/ml in IMPATH's test system.

e23 was found to have a very restricted pattern of reactivity. Positive reactivity was observed in tissues of simple, stratified and squamous epithelial origin. e23 showed positive reactivity with cells of the bladder, breast, cervix, esophagus, large and small intestines, bronchial and alveolar cells of the lung, parotid gland, prostate, skin and endometrium of the uterus. The reactivity with e23 was restricted to the membrane of these epithelial cells; the intensity of reactivity varied from equivocal to strong.

All cells of lymphoid origin including bone marrow, peripheral blood, lymph node, spleen, and tonsil were unreactive with the test antibody.

Immunoreactivity was also not seen in cells of neuroectodermal origin including those in the brain, spinal cord, and peripheral nerve. Mesenchymal elements such as skeletal and smooth muscle cells, endothelial cells, as well as polymorphonuclear inflammatory cells were negative with antibody e23. Stromal cells of possible mesenchymal origin present in the prostate were reactive with antibody e23.

In summary, the reactivity observed with antibody e23 was restricted to the membrane of simple, stratified, and squamous epithelial cells in normal human tissues. Thus, given its minimal reactivity with normal tissues, e23 and its corresponding single chain antibodies, or humanized antibodies derived therefrom, and antibodies having the immu-

nobinding characteristics thereof should be well suited for use as a diagnostic or therapeutic agent for patients exhibiting cancers associated by gp185^{erbB-2} overexpression which include certain highly malignant tumors, such as adenocarcinoma of the stomach, lung, breast and ovary.

The reactivity of e23 and e21 was then compared for a number of primate tissues. The results of this comparison are set forth on the following page. The results therein clearly demonstrate that e23 exhibits substantially lower reactivity with normal primate tissues than e21. Given its lower reactivity with normal tissues, it is expected that e23 should function as a better diagnostic and/or therapeutic agent than e21. In particular, it is expected that e23 should provide for fewer false positive diagnoses and lesser adverse reactions to normal human tissues.

Comparison of e23 and e21 Antibodies

	e23	e21
Western Blot	–	–
Immunoprecipitation	+	+

-continued

Comparison of e23 and e21 Antibodies

	e23	e21
ELISA	+	+
Immunohistochemistry Formalin Fixed	–	+
Immunohistochemistry Frozen	+	+
Cynomolgus and Baboon Esophagus	luminal edge cells of epithelium, membrane	epithelial cells, membrane Periferal nerve cells
Cynomolgus and Baboon Breast	delicate fibroblast staining	ductal epithelium 3+ keratinocytes (skin) fibroblasts 2+ perineurium 2+ endothelim 1–2+ endoneurium 1+
Cynomolgus brain	cerebellum - no staining cortex - no staining	cerebellum - no staining cortex - arachnoid 2+ - dura 2+
Cynomolgus bladder	+/–	+, Membrane

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 11

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 711 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(i x) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..711

(i x) FEATURE:
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(B) LOCATION: 162
(D) OTHER INFORMATION: /note= "Nucleotide 162 wherein G is K =G or T(U)."

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GGG GAG AAG GTC ACA ATG ACT TGC AGG GCC ACC CCA AGT GTA AGT TAC          96
Gly Glu Lys Val Thr Met Thr Cys Arg Ala Thr Pro Ser Val Ser Tyr
            20                  25                  30

ATG CAC TGG TAT CAG CAG AAG CCA GGA TCC TCC CCC AAA CCT TGG ATT         144
Met His Trp Tyr Gln Gln Lys Pro Gly Ser Ser Pro Lys Pro Trp Ile
        35                  40                  45

TAT ACC ACA TCC AAC CTG GCT TCT GGA GTC CCT GCT CGC TTC AGT GGC         192
Tyr Thr Thr Ser Asn Leu Ala Ser Gly Val Pro Ala Arg Phe Ser Gly
    50                  55                  60

GGT GGG TCT GGG ACC TCT TAC TCT CTC ACA GTC AGC AGA GTG GAG GCT         240
Gly Gly Ser Gly Thr Ser Tyr Ser Leu Thr Val Ser Arg Val Glu Ala
65                  70                  75                  80

GAA GAT GCT GCC ACT TAT TAC TGC CAG CAG TGG AGT CGT AGC CCA CCC         288
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Glu Asp Ala Ala Thr Tyr Tyr Cys Gln Gln Trp Ser Arg Ser Pro Pro
                85                  90                  95

ACG TTC GGA GGG GGG TCC AAG CTG GAA ATA AAA GGT TCT ACC TCT GGT      336
Thr Phe Gly Gly Gly Ser Lys Leu Glu Ile Lys Gly Ser Thr Ser Gly
            100                 105                 110

TCT GGT AAA TCT TCT GAA GGT AAA GGT GTG CAG CTG CAG GAG TCA GGA      384
Ser Gly Lys Ser Ser Glu Gly Lys Gly Val Gln Leu Gln Glu Ser Gly
        115                 120                 125

CCT GAG GTG GTG AAG CCT GGA GGT TCA ATG AAG ATA TCC TGC AAG ACT      432
Pro Glu Val Val Lys Pro Gly Gly Ser Met Lys Ile Ser Cys Lys Thr
    130                 135                 140

TCT GGT TAC TCA TTC ACT GGC CAC ACC ATG AAC TGG GTG AAG CAG AGC      480
Ser Gly Tyr Ser Phe Thr Gly His Thr Met Asn Trp Val Lys Gln Ser
145                 150                 155                 160

CAT GGA AAG AAC CTT GAG TGG ATT GGA CTT ATT AAT CCT TAC AAT GGT      528
His Gly Lys Asn Leu Glu Trp Ile Gly Leu Ile Asn Pro Tyr Asn Gly
                165                 170                 175

GAT ACT AAC TAC AAC CAG AAG TTC AAG GGC AAG GCC ACA TTT ACT GTA      576
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            180                 185                 190

GAC AAG TCG TCC AGC ACA GCC TAC ATG GAG CTC CTC AGT CTG ACA TCT      624
Asp Lys Ser Ser Ser Thr Ala Tyr Met Glu Leu Leu Ser Leu Thr Ser
        195                 200                 205

GAG GAC TCT GCA GTC TAT TAC TGT GCA AGG AGG GTT ACG GAC TGG TAC      672
Glu Asp Ser Ala Val Tyr Tyr Cys Ala Arg Arg Val Thr Asp Trp Tyr
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(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(i x) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..720

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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  1               5                  10                  15

AAG GTC ACC ATG ACC TGC AGT GCC AGC TCA AGT GTA AGT AAC ATG CAC       96
Lys Val Thr Met Thr Cys Ser Ala Ser Ser Ser Val Ser Asn Met His
            20                  25                  30

TGG TAT CAG CAG AAG TCA AGC ACC TCC CCC AAA CTC TGG GTT TAT GAC      144
Trp Tyr Gln Gln Lys Ser Ser Thr Ser Pro Lys Leu Trp Val Tyr Asp
        35                  40                  45

ACA TCC AAA CTG GCT TCT GGA GTC CCA GGT CGC TTC AGT GGC AGT GGG      192
Thr Ser Lys Leu Ala Ser Gly Val Pro Gly Arg Phe Ser Gly Ser Gly
    50                  55                  60

TCT GGA AAC TCT TAC TCT CTC ACG ATC AGC AGC ATG GAG GCT GAA GAT      240
Ser Gly Asn Ser Tyr Ser Leu Thr Ile Ser Ser Met Glu Ala Glu Asp
65                  70                  75                  80

GCT GCC ACT TAT TAT TGT TAT CAG GGG AGT GGG TAC CCA TTC ACG TTC      288
Ala Ala Thr Tyr Tyr Cys Tyr Gln Gly Ser Gly Tyr Pro Phe Thr Phe
                85                  90                  95
```

-continued

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GGC TCG GGG ACA AAG TTG GAA ATA AAA GGT TCT ACC TCC GGA TCT GGT      336
Gly Ser Gly Thr Lys Leu Glu Ile Lys Gly Ser Thr Ser Gly Ser Gly
                100                 105                 110

AAA TCT TCT GAA GGT AAA GGT GTG CAG CTG CAG CAG TCT GGG GTT GAG      384
Lys Ser Ser Glu Gly Lys Gly Val Gln Leu Gln Gln Ser Gly Val Glu
            115                 120                 125

CTT GTC CGA GGA GGG GCC TTA GTC AAG TTG TCC TGC AAA GCT TCT GAC      432
Leu Val Arg Gly Gly Ala Leu Val Lys Leu Ser Cys Lys Ala Ser Asp
        130                 135                 140

TTC AAC ATT AAA GAC TAT TAT ATC CAC TGG GTG AAG CAG AGG CCT GAA      480
Phe Asn Ile Lys Asp Tyr Tyr Ile His Trp Val Lys Gln Arg Pro Glu
145                 150                 155                 160

CAG GGC CTG GAA TGG ATT GGA TGG ATT CAT CCT GAG AAT GGT AAT ACT      528
Gln Gly Leu Glu Trp Ile Gly Trp Ile His Pro Glu Asn Gly Asn Thr
                165                 170                 175

GTA TAT GAC CCG AAA TTC CAG GGC AAG GCC AGT ATA ACA GCA GAC ACA      576
Val Tyr Asp Pro Lys Phe Gln Gly Lys Ala Ser Ile Thr Ala Asp Thr
            180                 185                 190

TCC TCC AAC GCG GCC TAC CTT CAG CTC AGC AGC CTG ACA TCT GAG GAC      624
Ser Ser Asn Ala Ala Tyr Leu Gln Leu Ser Ser Leu Thr Ser Glu Asp
        195                 200                 205

ACT GCC GTC TAT TAC TGT GCT TCT TAT TAC TAC TAT AGT GCT TAC TAT      672
Thr Ala Val Tyr Tyr Cys Ala Ser Tyr Tyr Tyr Tyr Ser Ala Tyr Tyr
    210                 215                 220

GCT ATG TAC TAC TGG GGT CAA GGA ACC TCG GTC ACC GTC TCC TCA TAA      720
Ala Met Tyr Tyr Trp Gly Gln Gly Thr Ser Val Thr Val Ser Ser
225                 230                 235                 240
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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(2) INFORMATION FOR SEQ ID NO:5:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(i x) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 21

-continued

(D) OTHER INFORMATION: /note= "N =inosine."

(i x) FEATURE:
(A) NAME/KEY: misc_feature
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(D) OTHER INFORMATION: /note= "W =A or T."

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

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(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:9:

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(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

-continued

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:10:

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(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Gly Ser Thr Ser Gly Ser Gly Lys Ser Ser Glu Gly Lys Gly
1 5 10

We claim:

1. A single chain antibody which binds to erbB-2 which comprises the variable heavy and light domains of a monoclonal antibody selected from the group consisting of e21 having ATCC Accession No. HB11602 and e23 having ATCC Accession No. HB11601.

2. The single chain antibody of claim **1** which comprises the amino acid sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2.

3. An immunoconjugate comprising the single chain antibody of claim **1** bound to a cytotoxic moiety or a label.

4. An immunoconjugate comprising the single chain antibody of claim **2** bound to a cytotoxic moiety or a label.

5. The immunoconjugate of claim **3** or claim **4** wherein said label is selected from the group consisting of a fluorescent label, an enzymatic label, and a radiolabel.

6. A diagnostic composition suitable for in vitro assaying for $gp185^{erbB-2}$ which comprises a diagnostically effective amount of at least one of the single chain antibodies according to claim **1**, in a carrier.

7. A diagnostic composition suitable for in vitro assaying for $gp185^{erbB-2}$ which comprises a diagnostically effective amount of the immunoconjugate of claim **3**, in a carrier.

8. The diagnostic composition of claim **7**, wherein the single chain antibody comprises the amino acid sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2.

9. The diagnostic composition of claim **6**, wherein the single chain antibody comprises the amino acid sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2.

10. The diagnostic composition of claim **6**, wherein the single chain antibody is fused to an enzyme or fragment or derivative thereof comprising enzymatic activity.

11. The diagnostic composition of claim **10**, wherein the enzyme comprises horseradish peroxdase or alkaline phosphatase.

12. The diagnostic composition of claim **6**, wherein the single chain antibody comprises the amino acid sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2.

* * * * *

Exhibit 2

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Antibody-Mediated Delivery of Tumor Necrosis Factor (TNF-α): Improvement of Cytotoxicity and Reduction of Cellular Resistance

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(Submitted October 16, 1990; sent for revision October 26; received October 31; accepted November 5, 1990)

Abstract. Recombinant human tumor necrosis factor-α (TNF-α) is a macrophage-derived, non-glycosylated (17 kDa) peptide that has a remarkably broad range of biological and immunological effects including antiviral action and cytotoxic and cytostatic effects. TNF-α was coupled to murine antibody ZME-018, which recognizes a 240 kDa glycoprotein present on over 80% of melanoma cells. The crosslinking was accomplished using the heterobifunctional crosslinking reagent, *N*-succimindyl 3-(2-pyridyldithio) proprionate (SPDP). After purification on gel-permeation and affinity columns, the resulting eluate was analyzed by non-reducing SDS-PAGE, which confirmed that the product was a mixture of ZME-018 coupled to one or two TNF-α molecules. The ZME-TNF conjugate was titered against murine L-929 cells to demonstrate the presence of active TNF. ELISA of the conjugate against target BRO human melanoma cells or non-target T-24 cells demonstrated specific binding only to target cells. Melanoma BRO cells were killed by the immunoconjugate (IC_{50} of 10 units/mL), whereas native TNF-α had no effect at concentrations >50,000 units/mL. The immunoconjugate and TNF-α were inactive against T-24 non-target cells. These studies suggest that the sensitivity of cells to TNF was dramatically augmented by specific antibody mediated delivery to tumor cells.

Murine monoclonal antibodies to human tumor cell surface antigens have the unique ability to distribute to and accumulate within human tumors after systemic administration [1–5]. Numerous efforts have been made to exploit the selectivity and specificity of these reagents for cancer therapy [6–10]. Radiolabeled antibodies to human melanoma antigens [11, 12], to CEA[c] [13, 14], and to other cell-surface antigens [1, 15–19] exhibit specific tumor localization in patients after administration.

Murine antibody ZME-018 binds to epitope "a" of a high molecular weight antigen (gp240) found on the surface of more than 80% of melanoma cell lines and fresh tumor samples [20]. When labeled with ^{111}In and administered systemically to patients with melanoma, ZME-018 has been found to localize in 77% of soft tissue melanoma lesions [21].

Because of their unique abilities to localize within human tumors after systemic administration, antibodies have the potential to serve as targeting vehicles for specific delivery of cytotoxic chemotherapeutic agents, toxic peptides, biological response modifiers, and therapeutic radionuclides.

TNF-α is a polypeptide secreted primarily by activated macrophages [22], and it shares approximately 30% structural homology with another peptide hormone, LT (also termed TNF-β), that is secreted by activated lymphocytes [23]. Studies by Beutler et al. [24], Carswell et al. [25], and others [26, 27] have implicated TNF-α along with the other peptide hormones as one of those responsible for the mediation of the mammalian response to bacterial endotoxin. Considerable evidence also exists that TNF-α may play a role in the tumoricidal activity of activated macrophages [28]. Recently, human TNF-α was purified, sequenced, and cloned by recombinant DNA techniques [29]. Purified human recombinant TNF-α is a single-chain, non-glycosylated polypeptide with a molecular weight of 17.1 kDa [30]. *In vitro*, TNF-α is cytostatic or cytotoxic to a number of mammalian and human tumor cells [31]. Tusujimoto et al. [32] and Baglioni et al. [33] demonstrated that human cells in culture express high affinity (kDa of 10^{-9} to 10^{-10}) binding sites for TNF-α. Cells have been shown to contain between 100 and 5,000 receptors per cell; there has been, however, no apparent correlation between the number (or affinity) of receptors per cell and the cellular response to TNF-α antiproliferative effects [34], suggesting a mechanism beyond receptor signaling that accounts for TNF-α action.

Recent clinical studies with TNF-α were disappointing [35–38] due to the inability to achieve tumoricidal levels of TNF-α in serum and the restricted cytotoxicity of this molecule. Antibody conju-

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[c]*Abbreviations used:* CEA, carcinoembryonic antigen; TNF-α, tumor necrosis factor; LT, lymphotoxin; IFN-α, α-interferon; SPDP, *N*-succimindyl 3-(2-pyridyldithio) proprionate; ABTS, 2,2′-azino-bis (3-ethyl benzthia zoline-6 sulfonic acid); EDTA, ethylenediamine tetraacetic acid; DMF, dimethyl formamide; MEM, minimum essential medium; FBS, fetal bovine serum; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate buffered saline; Buffer A, 100 mM sodium phosphate, pH 7.0, and 0.5 mM EDTA; TEA-HCl, triethanolamine hydrocholoride; Buffer B, 5 mM bis-Tris acetate buffer, pH 5.8, 50 mM NaCl, and 1 mM EDTA; SDS-PAGE, sodium dodecyl sulfate polyacrylimide gel electrophoresis; ZME-TNF conjugate, conjugate of murine antibody ZME-018 and TNF-α; BSA, bovine serum albumin.

gates have the ability to modify the natural pharmacology and therapeutic efficacy of proteinaceous biological-response modifiers, such as IFN-α [39] and TNF-α. Accordingly, we have coupled recombinant human TNF-α to the murine antimelanoma monoclonal antibody ZME-018 using the heterobifunctional crosslinking reagent SPDP as a model system to evaluate the utility of antibody-guided delivery of endogenous cytotoxic cytokines.

MATERIALS AND METHODS

Materials. The reagents 2-iminothiolane, ABTS, and SPDP were obtained from the Sigma Chemical Co. (St. Louis, MO). EDTA (disodium salt) was purchased from Boehringer Manneheim Corp. (Indianapolis, IN). TEA-HCl was obtained from the Kodak Chemical Co. (Rochester, NY) and DMF was purchased from the Aldrich Chemical Co. (Milwaukee, WI). Tris buffer was obtained from Bio-Rad Laboratories, Inc. (Richmond, CA). Recombinant human tumor necrosis factor (0.5 mg/mL, sp. act. 3×10^7 units/mL) was obtained from Genentech Inc. (San Francisco, CA). Antibody ZME-018 was a gift from Hybritech, Inc. (San Diego, CA). Dulbecco's modified Eagle's medium was obtained from Cellgro, Inc. (Washington, D.C.). MEM was obtained from GIBCO Laboratories (Grand Island, NY). FBS was purchased from Hyclone, Inc. (Logan, UT). Horseradish-peroxidase conjugated goat antimouse antibody for ELISA was purchased from Bio-Rad Laboratories.

Cells. Human melanoma cells A375-M and AAB-527 were obtained from Dr. I. J. Fidler of the M. D. Anderson Cancer Center (Houston, TX) and Dr. Pat Trown of Xoma Corp. (Davis, CA), respectively. The A375-M cells were routinely grown at a density of 7×10^6 cells per T-75 flask in Dulbecco's MEM 10% FBS that contained gentamicin (0.05 mg/mL), added sodium pyruvate (100 mM), non-essential amino acids (10 mM), glutamine (200 mM), and MEM vitamins. The cells were routinely subcultured twice each week. The AAB-527 cells were grown in Dulbecco's MEM 10% FBS that contained gentamicin (0.05 mg/mL) and added sodium pyruvate (100 mM). Murine L-929 cells were purchased from the American Type Culture Collection (Rockville, MD) and were grown in Dulbecco's MEM 10% FBS that contained gentamicin (0.05 mg/mL) and glutamine (200 mM).

All cells were routinely tested and found to be free of *Mycoplasma* contamination using the Gen-Probe assay kit (Fisher Scientific, Houston, TX).

Conjugation of ZME-018 with TNF-α. ZME-018 (2 mL, 4.85 mg/mL) in PBS was placed in a 12 × 75 mm glass tube; a solution of SPDP (6 mg/mL) in dry DMF was added, while being vortexed with a 5-fold molar excess of SPDP. The mixture was incubated for 30 min at room temperature, and excess unreacted SPDP was removed by gel filtration on a Sephadex G-25 column (2 × 30 cm) equilibrated with Buffer A. The void-volume peaks, which contained protein, were pooled and kept on ice.

TNF-α (5 mg, 0.5 mg/mL) was added to TEA-HCl and EDTA to a final concentration of 60 mM TEA-HCl, and 1 mM EDTA, pH 8.0; 2-iminothiolane was added to a final concentration of 5 mM. The mixture was incubated for 90 min at 4°C under a stream of N_2. Excess iminothiolane was removed by gel filtration on a Bio-Rad P-6 column (2 × 40 cm) pre-equilibrated with Buffer B. The modified TNF-α that eluted in the void volumes were pooled and kept at 4°C.

Modified ZME-018 in Buffer A was mixed with modified TNF-α in Buffer B in a molar ratio of 1:10. The pH of the mixture was adjusted to 7.0 by the addition of 0.5 M TEA-HCl, pH 8.0, and the mixture was incubated for 20 hr at 4°C. The reaction mixture was purified by chromatography on Sephacryl S-300HR. High molecular weight fractions were pooled and further purified by chromatography on an affinity column.

The affinity support for TNF-α was prepared by first dialyzing 300 μg of anti-TNF-α murine antibody (TNF-β, obtained from Genentech Inc.) in 0.1 M $NaHCO_3$ buffer, pH 8.5. Affigel-10 resin (1 mL) from Bio-Rad Laboratories was transferred to a 5 mL centrifuge tube and washed three times with cold (4°C) deionized water. The antibody and the resin were admixed and gently agitated at 4°C for 24 hr. The resin was washed, and the remaining active ester groups were blocked by the addition of 1 mL of 0.1 M $NaHCO_3$, pH 8.0, and 0.1 mL of 1 M ethanolamine.

The high molecular weight fractions from S-300 chromatography (fractions 35–55) were pooled and applied to an anti-TNF-α affigel column (1 cm × 5 cm) equilibrated with PBS. The sample was loaded and washed with 50 mL of PBS to elute free antibody. TNF-α, conjugated to ZME-018, was eluted by the addition of 50 mM sodium citrate buffer, pH 3.0, which contained 150 mM NaCl. The column effluent was fractionated using a Gilson fraction collector (model FC-80). Protein analysis of the various fractions was performed using a Bio-Rad protein assay. Fractions from the various steps were analyzed by silver-stained SDS-PAGE using a 5–15% acrylamide gradient gel.

ELISA of ZME-018 and ZME-TNF conjugate. An ELISA was performed to determine the immunoreactivity of the ZME-TNF conjugate compared to ZME-018 alone. Briefly, 5×10^4 melanoma cells (A375-M) per well were added to 96-well polyvinyl microtiter plates (Falcon Plastics, Inc., Oxnard, CA). The plates were dried for 18 hr at 37°C and then washed twice with 10 mM PBS, pH 7.4, that contained 0.1% Tween-20 and 0.02% Thimersol (washing buffer). Antibody ZME-018 or the ZME-TNF conjugate was diluted in washing buffer that contained 0.1% BSA, and then added to each well. The plates were incubated for 1 hr at room temperature. After three washes with washing buffer, 50 μL of a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad Laboratories) was added to each well,

which were incubated for 1 hr at room temperature. After another three washes, 100 μL of substrate (1 mM ABTS), which contained 1 μL/mL of 3% hydrogen peroxide was added to each well. The reaction was stopped after 10–20 min by the addition of 5% SDS in PBS; absorbance at 405 nm was measured with an ELISA reader.

In vitro cytotoxicity of TNF-α and ZME-TNF conjugate. To examine the cytotoxicities of ZME-TNF conjugate and TNF-α, antigen-positive human melanoma cells (A375-M or AAB-527) in MEM with 10% FBS were plated on 96-well plates at a density of 5 x 10^3 cells per well and were allowed to adhere for 24 hr at 37°C in 5% CO_2. After 24 hr, the medium was replaced with medium that contained various concentrations of either TNF-α or the ZME-TNF conjugate.

Measurements of the effects of TNF-α and ZME-TNF conjugate on cell growth. The effects on the growth of tumor cells in culture were determined by crystal violet staining. After additional incubation for 72 hr at 37°C, the medium was aspirated, and the cell monolayers were rinsed three times with Ca^{2+}- and Mg^{2+}- free PBS. Following the final rinse, wells were tapped dry, and cells were fixed and stained by the addition of 0.5% crystal violet in 20% methanol (0.1 mL/well). Following a 10 min incubation, plates were rinsed three times in deionized water, and crystal violet was extracted from adherent cells by the addition of 0.2 mL of Sorenson's buffer per well (0.1 M sodium citrate, pH 4.2, in 50% ethanol). Cell plates were vortexed for 30 min at room temperature, and the absorbance was read at 540 nm (Model EL-309, Bio-Tek Instruments, Winooski, VT) and compared with control wells (medium alone). All values shown are means of duplicate experiments performed in octuplicate.

RESULTS

Conjugation of ZME-018 with TNF-α. Once the coupling procedure was completed, the ZME-TNF conjugate reaction mixture was applied to an S-300 gel permeation column. As shown in Figure 1A,

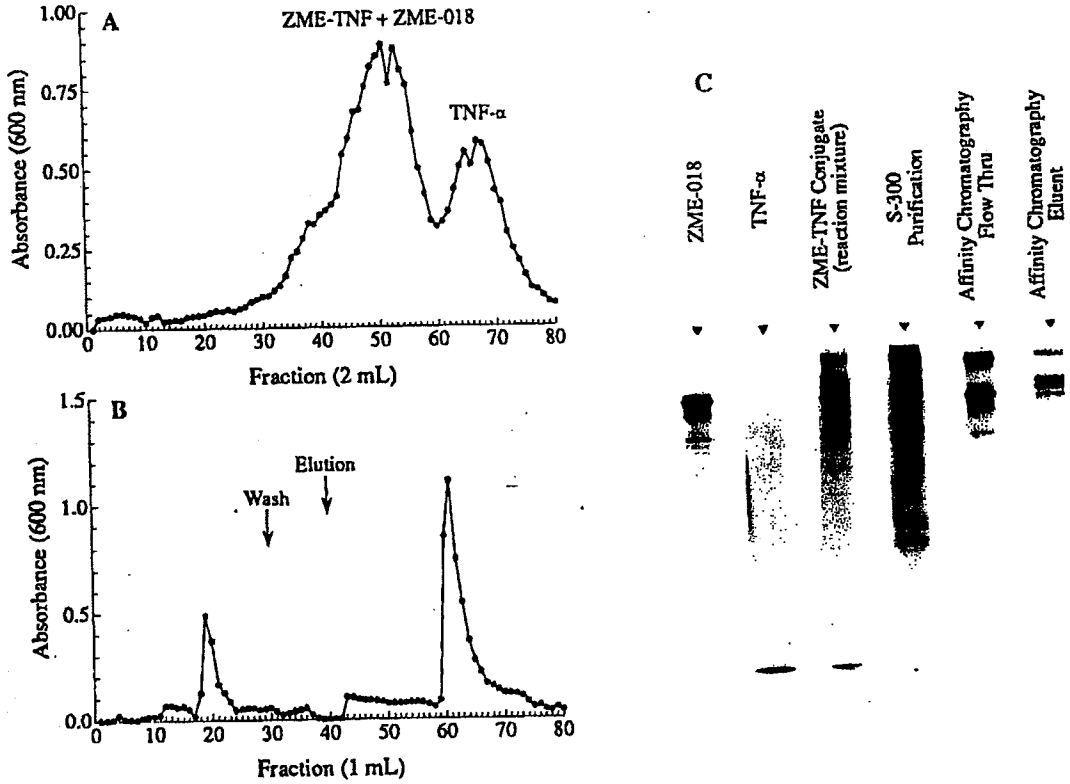

Figure 1. Analysis of the ZME-TNF conjugate reaction mixture. (A) The reaction mixture was first purified by chromatography on Sephacryl S-300HR. The two major peaks composed of ZME-018 and ZME-TNF conjugate (fractions 35–55) and free TNF-α (fractions 60–75) were separated. (B) The S-300 high molecular-weight peaks (fractions 35–55) were pooled and applied to an Affigel 10 column coupled to an anti-TNF-α antibody and equilibrated with PBS. The sample was loaded and washed with PBS to elute free ZME-018. TNF-α conjugated to ZME-018 was eluted by the addition of 50 mM sodium citrate buffer that contained 150 mM NaCl, pH 3.0. Protein analysis for collected fractions exhibited a flow-through peak (fractions 16–25) and a bound peak (fractions 59–70). (C) Fractions from the various purification steps were analyzed by silver-stained SDS-PAGE using a 5–15% acrylamide gradient gel. The ZME-TNF conjugate reaction mixture (lane 3) shows the presence of free ZME-018 and unreacted TNF-α. The high molecular-weight fraction from S-300 chromatography demonstrates the presence of free ZME-018 antibody and high molecular-weight conjugates essentially free of unreacted TNF-α. The major peak from the affinity chromatography flow-through (lane 5) contained free ZME-018 antibody and high molecular-weight component while the bound material (lane 6) was composed primarily of ZME-018 conjugated to one or two TNF-α molecules, with lesser amounts of free ZME-018.

analysis of the column effluent revealed two major peaks: a high molecular weight peak composed of free ZME-018 antibody and the ZME-TNF conjugate (fractions 35–55) and a lower molecular weight peak (fractions 60–75) composed of free TNF-α. Analysis of the pooled high molecular weight peaks of SDS-PAGE (Figure 1C) demonstrated the presence of free ZME-018 antibody and of a high molecular weight conjugate essentially free of TNF-α. Application of the high molecular weight conjugates to an anti-TNF-α affinity column (Figure 1B) demonstrated a flow-through peak that was composed primarily of ZME-018 antibody and a high molecular weight conjugate (Figure 1C).

After purification using affinity chromatography, the final product was found by SDS-PAGE to be composed of ZME-018 coupled to one TNF-α molecule (major), ZME-018 bound to two TNF-α molecules (minor), with lesser amounts of free ZME-018 (Figure 1C, lane 6). There was no detectable free TNF-α in the final preparation. The final product, however, as shown in Figure 1C also appeared to contain small amounts of a high molecular weight material. This material may have represented a small amount of antibody-TNF-α aggregates.

To determine whether the functionality of the ZME-018 antibody was compromised by covalent coupling to TNF-α, the binding of native ZME-018 and ZME-TNF conjugate to antigen-positive melanoma (A325-M) cells was assessed by ELISA. As shown in Figure 2, both ZME-018 and the ZME-TNF conjugate bound to target cells to the same extent. Neither ZME-018 nor ZME-TNF conjugate bound detectably to non-target human bladder carcinoma (T-24) cells (data not shown).

To determine the functionality of the TNF-α component of the ZME-TNF conjugate, a TNF-α bioassay was performed as previously described [25]. One unit of activity is defined as the amount of protein which will cause 50% cytotoxicity to murine L-929 cells. The activity of the final preparation was 0.6×10^6 units/mg protein. This activity translated into a final activity of 0.11×10^{15} units/mol of ZME-TNF conjugates (assuming an average molecular weight of 180 kDa). Thus, the biological activity of the ZME-TNF conjugate against L-929 cells was approximately 10-fold less than that of native recombinant TNF-α (10^{15} units/mol). Preliminary studies prior to conjugation demonstrated that chemical modification of TNF-α with the 2-iminothiolane reagent reduced the biological activity of the molecule 2-fold. Thus the reduction in biological activity of the ZME-TNF conjugate may have been due to steric factors that accompany proximity to the large immunoglobulin molecules. The cytotoxicity of the ZME-TNF conjugate was assayed against log-phase antigen-positive human melanoma (A375-M) cells in culture. A 50% growth inhibitory effect was obtained with a concentration of 30 units/mL of TNF-α alone (Figure 3). In contrast, a 10-fold lower concentration (3 units/mL) of ZME-TNF conjugate was required to achieve the same effect. Thus, against antigen-positive cells, ZME-TNF conjugate was 10-fold more active on a unit basis than native TNF-α. On a molar basis, however, the IC_{50} for TNF-α of 30 units/mL translates into a 30×10^{-12} M concentration, and the IC_{50} for ZME-TNF conjugate of 3 units/mL translates into a concentration of 27×10^{-12} M.

Maximal binding of ZME-018 antibody to target cells occurred within 2 hr of exposure at 37°C (data not shown). Continuous exposure, however, of cells to TNF-α itself for 12–18 hr was required to attain an optimal cytotoxic effect. If ZME-018 antibody binding to melanoma cells mediates the cytotoxicity of the ZME-TNF conjugate, cytotoxicity of ZME-TNF conjugate should occur with minimal exposure. A 2 hr pulse exposure of ZME-TNF conjugate or TNF-α to A375-M melanoma cells (Figure 4) demonstrated no appreciable cytotoxicity (~10%) of TNF-α under these conditions at concentrations up to 2,000 units/

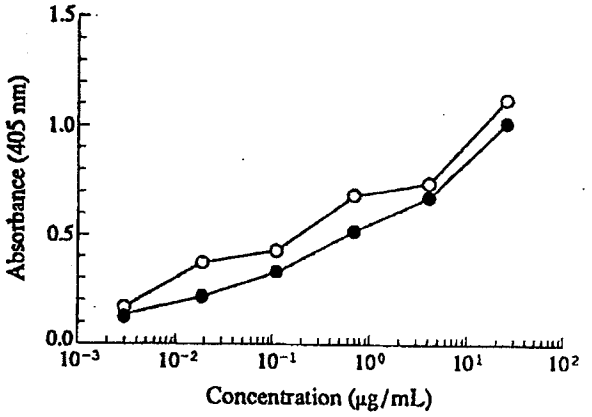

Figure 2. Determination by ELISA of the immunoreactivity of the ZME-TNF conjugate compared with ZME-018 alone in antigen positive melanoma (A325-M) cells. Both ZME-018 and ZME-TNF conjugate bound to antigen-positive A375-M melanoma cells. Key: ZME-018 (○), ZME-TNF conjugate (●).

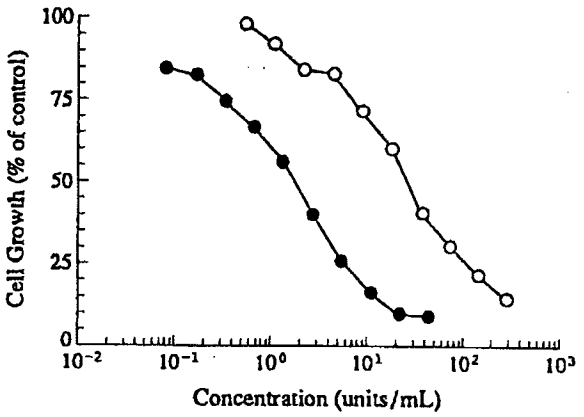

Figure 3. Cytotoxicity of ZME-TNF conjugate and TNF-α against antigen-positive human melanoma cells (A375-M). The cells were incubated for 72 hr and analyzed for relative cell proliferation by crystal violet staining. Values shown are the means of eight duplicate experiments. Key: ZME-TNF conjugate (●), TNF-α (○).

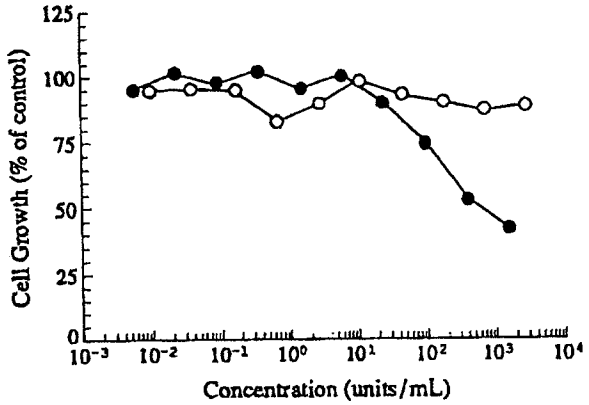

Figure 4. Pulse-exposure of TNF-α or ZME-TNF conjugate against A375-M cells. The cytotoxicity of the ZME-TNF conjugate and TNF-α alone was evaluated against log-phase human melanoma (A375-M) cells treated for 2 hr with either agent. This experiment was performed as described in Figure 3 except that 2 hr after treatment with TNF-α or ZME-TNF conjugate, the medium was removed, and the cells were washed two times with fresh medium and incubated for an additional 70 hr. Cytotoxicity was evaluated using the crystal violet assay. Key: TNF-α (○), ZME-TNF conjugate (●).

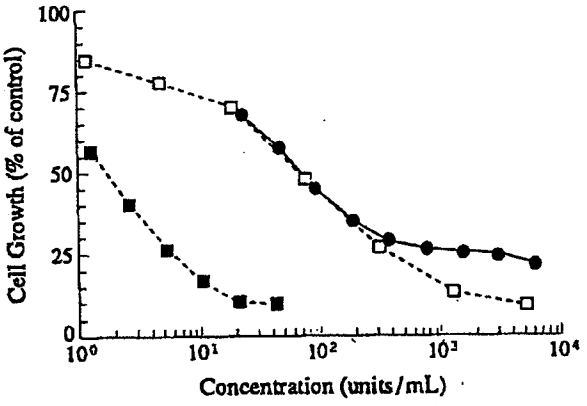

Figure 5. Effect of ZME-018 addition on the cytotoxicity of ZME-TNF conjugate. A continuous-exposure cytotoxicity assay using various doses of TNF-α or ZME-TNF conjugate was performed as described in Figure 3. Key: ZME-TNF conjugate alone (■), TNF-α alone (□), ZME-TNF conjugate + 50 μg/mL of ZME-018 (●).

mL. In contrast, ZME-TNF conjugate demonstrated 60% cytotoxicity after a 2 hr exposure of the highest dose (2,000 units/mL) tested. These studies demonstrate that the ZME-TNF conjugate required a shorter cellular exposure duration than TNF-α alone, most probably because of antibody-mediated interaction of TNF-α with either intracellular or cell-surface components.

To further examine the antibody-mediated cytotoxicity of the ZME-TNF conjugate, concentration-response curves for ZME-TNF conjugate and TNF-α were generated against antigen-positive human melanoma cells in the presence or absence of either ZME-018 or an irrelevant, murine antibody (Figure 5). In the presence of irrelevant antibody, the ZME-

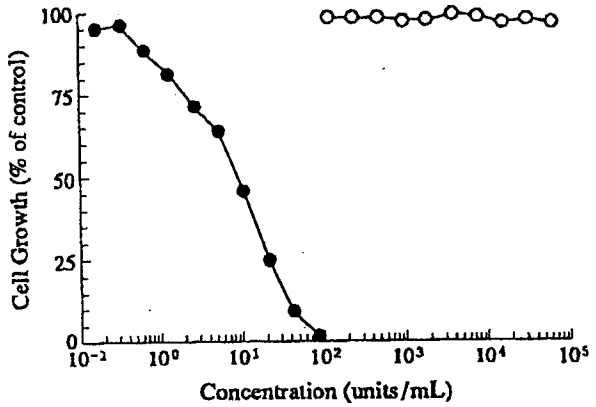

Figure 6. The cytotoxicity of ZME-TNF conjugate evaluated against an antigen-positive, TNF-α-resistant, human melanoma cell line (AAB-527). A 72 hr continuous-exposure cytotoxicity assay was performed as described in Figure 3 using log-phase AAB-527 cells. Key: ZME-TNF conjugate (●), TNF-α (○).

TNF conjugate exhibited 50% cytotoxicity (IC_{50}) at a concentration of 2 units/mL. In the presence of free ZME-018 (50 μg/mL), the cytotoxicity of the ZME-TNF conjugate was suppressed and generated a concentration-response curve similar to that of TNF-α alone. Thus, the augmented cytotoxicity of the ZME-TNF conjugate compared with TNF-α alone appeared to be mediated by specific binding to the cell surface antigen recognized by the ZME-018 antibody.

The cytotoxic effects of the ZME-TNF conjugate were tested against various cell types. Against TNF-α-resistant antigen-negative bladder carcinoma (T-24) cells, the ZME-TNF conjugate did not demonstrate growth-modulating effects (cytotoxic or growth-stimulatory). However, against antigen-positive TNF-α-resistant human melanoma (AAB-527) cells (Figure 6), the ZME-TNF conjugate demonstrated 50% cytotoxicity at a concentration of 10 units/mL. At a concentration of 100 units/mL, the ZME-TNF conjugate showed more than 95% growth inhibition against target cells. In contrast, TNF-α alone at concentrations of up to 100,000 units/mL showed no cytotoxic effects. Therefore, the ZME-TNF conjugate demonstrated impressive growth inhibition effects against cells resistant to TNF-α alone. Since this effect was observed only with antigen-positive cells, the data support the hypothesis that the observed increase in cytotoxicity was mediated by the binding of ZME-018 antibody to the cell surface.

DISCUSSION

The ability of a murine antibody to augment the cytotoxic properties of TNF-α action has several possible explanations. The antibody may hold TNF-α at the cell membrane in proximity to the TNF-α receptor and, thus, result in improved recognition of the TNF-α ligand by the TNF-α receptor. Alternatively, the interaction of the ZME-TNF conjugate with the TNF-α receptor may differ from that of TNF-α alone and may modulate or interfere with the nominal cel-

lular processing of the TNF-α receptor-ligand complex [40]. On the other hand, internalization of active TNF-α via the ZME-TNF conjugate complex may allow interaction of TNF-α ligand with intracellular receptors for TNF-α and may generate an intracellular cytotoxic signal.

Our group [41] and others [42, 43] have shown that IFN-α can also be successfully coupled to murine monoclonal antibodies to create an antiviral and cytotoxic agent that is more selective and effective than free IFN-α. Since biological response modifiers such as TNF-α and IFN-α may suffer from poor tumor selectivity and a short biological half-life, coupling to monoclonal antibodies may confer selectivity and augmented sensitivity to otherwise non-specific cytotoxic agents.

If *in vivo* targeting studies demonstrate localization of the ZME-TNF conjugate within tumors, this reagent may be clinically useful for tumor-targeted delivery of the biological response modifier TNF-α. In addition, antibody-mediated delivery of biological response modifiers may, in general, provide a new generation of tumor-directed agents with improved selectivity and greater biological activity.

ACKNOWLEDGEMENT: The research was conducted, in part, by the Clayton Foundation for Research, Houston, Texas.

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Exhibit 3

US005650150A

United States Patent [19]

Gillies

[11] Patent Number: 5,650,150

[45] Date of Patent: Jul. 22, 1997

[54] **RECOMBINANT ANTIBODY CYTOKINE FUSION PROTEINS**

[76] Inventor: **Stephen D. Gillies**, 245 Leavitt St., Hingham, Mass. 02043

[21] Appl. No.: **281,238**

[22] Filed: **Jul. 27, 1994**

Related U.S. Application Data

[63] Continuation of Ser. No. 788,765, Nov. 7, 1991, abandoned, which is a continuation-in-part of Ser. No. 612,099, Nov. 9, 1990, abandoned.

[51] **Int. Cl.**[6] **A61K 39/395**; A61K 39/40; A61K 45/05; C12P 21/04

[52] **U.S. Cl.** **424/134.1**; 424/133.1; 424/85.1; 435/69.7

[58] **Field of Search** 530/387.1, 387.3, 530/388.3, 387.8; 424/134.1, 133.1, 159.1, 147.1, 155.1; 435/69.7

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Primary Examiner—Lila Feisee
Assistant Examiner—Ray F. Ebert
Attorney, Agent, or Firm—Testa, Hurwitz & Thibeault, LLP

[57] **ABSTRACT**

Immunoconjugates for the selective delivery of a cytokine to a target cell are disclosed. The fusion proteins are comprised of an immunoglobulin heavy chain having a specificity for the target cell, such as a cancer or virus-infected cell, and a cytokine, such as lymphotoxin, tumor necrosis factor alpha, interleukin-2, or granulocyte-macrophage colony stimulating factor, joined via its amino terminal amino acid to the carboxy-terminus of the immunoglobulin. Nucleic acid sequences encoding these fusion proteins and methods of their preparation by genetic engineering techniques are also disclosed.

21 Claims, 9 Drawing Sheets

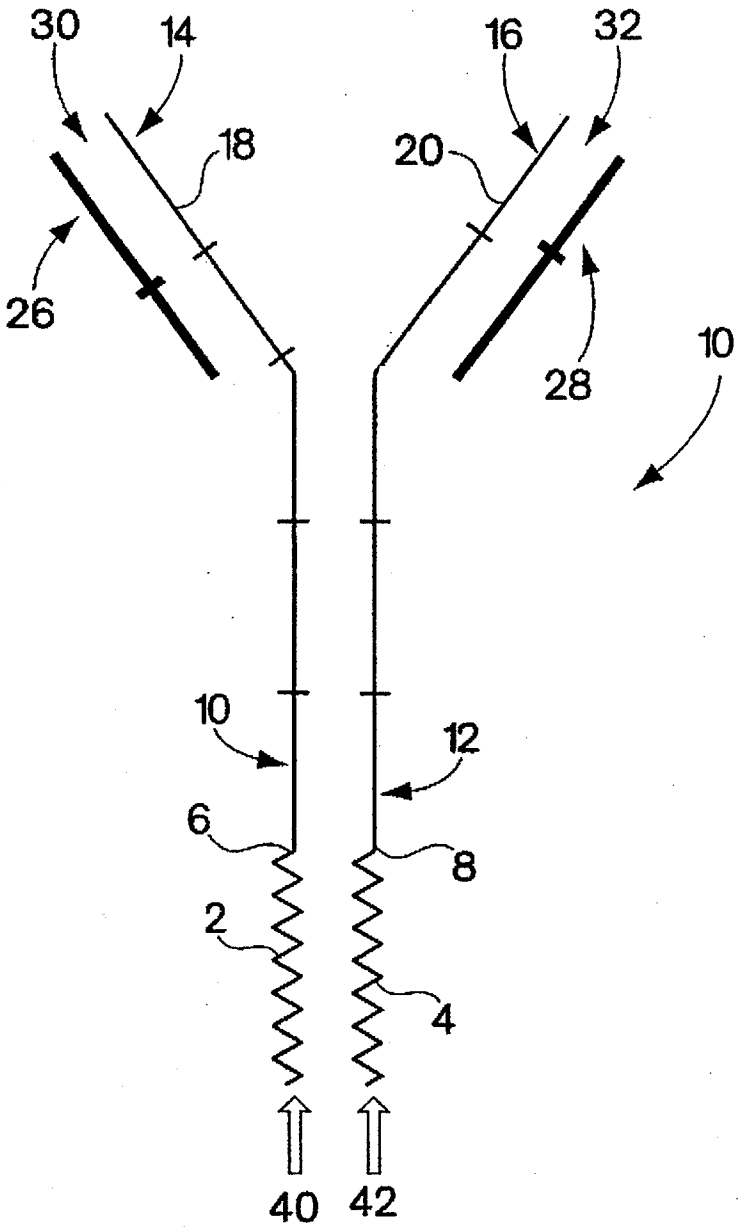


Fig. 1

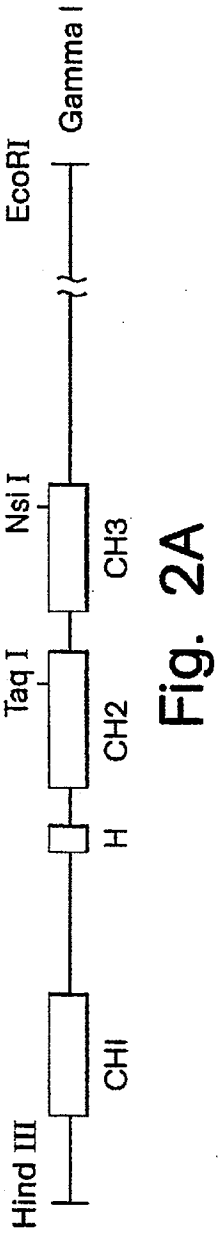


Fig. 2A

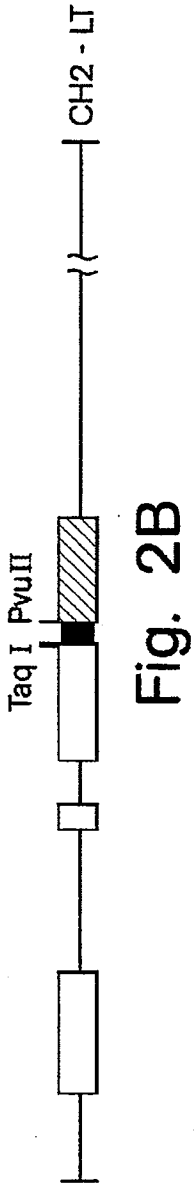


Fig. 2B

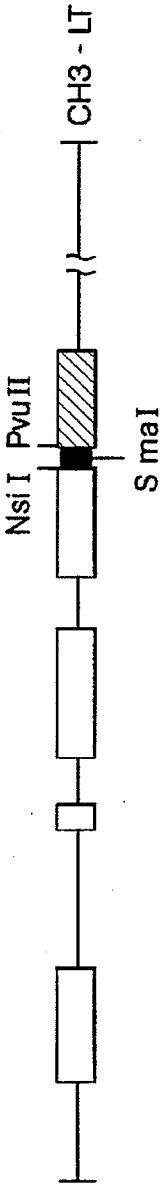


Fig. 2C

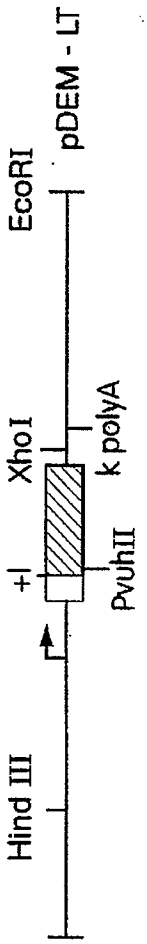


Fig. 2D

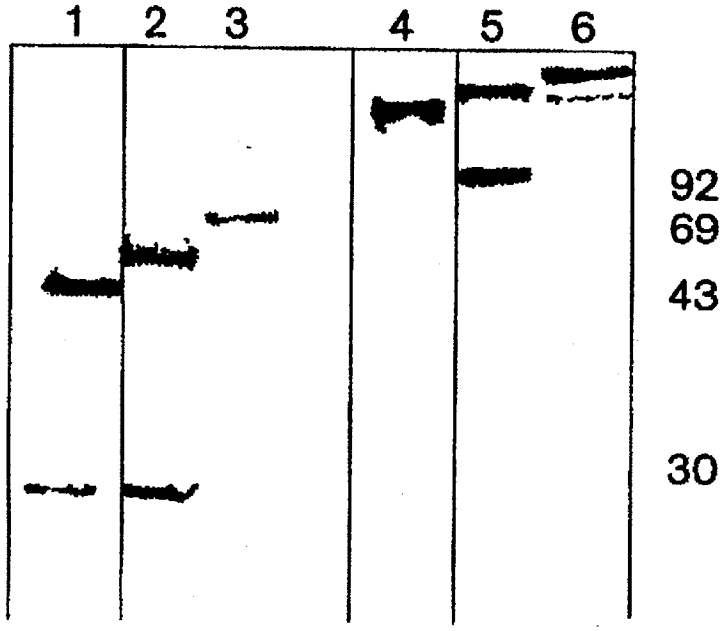


Fig. 3

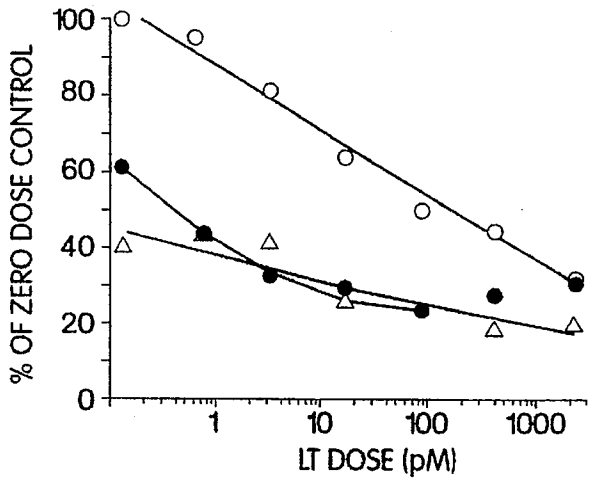


Fig. 4A

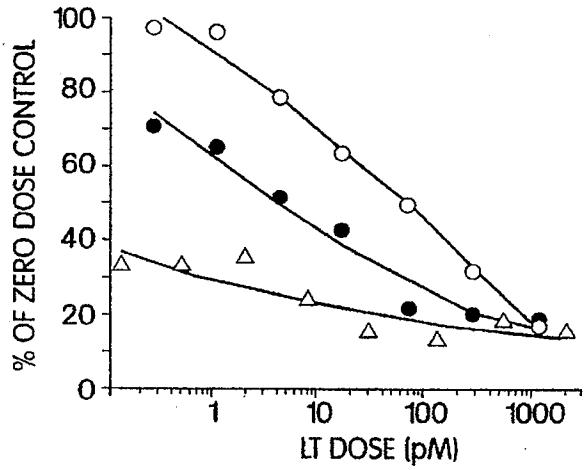
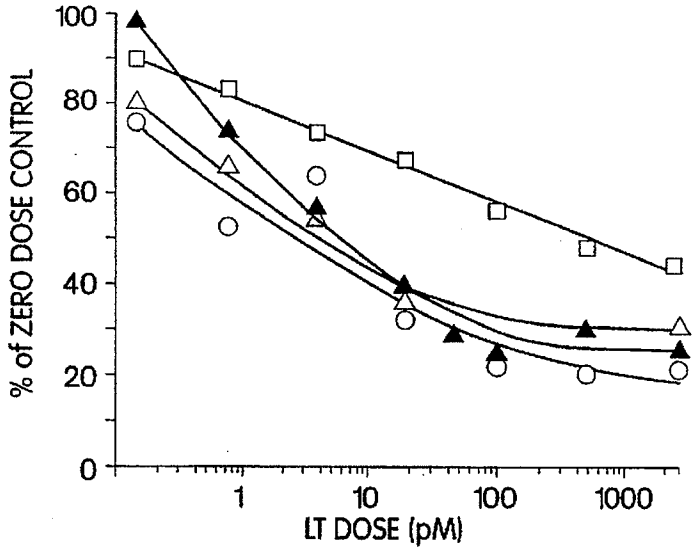


Fig. 4B



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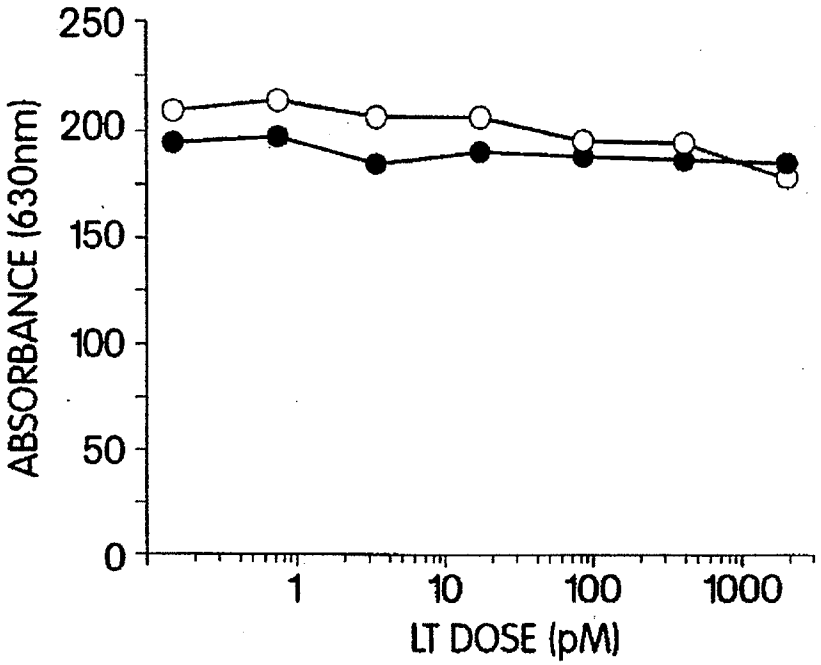
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Fig. 6A

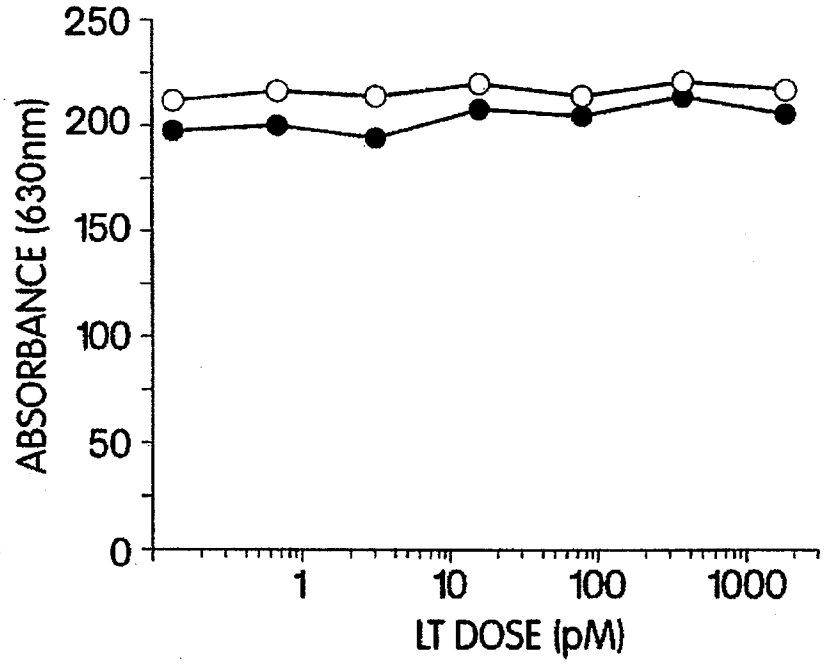
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Fig. 6B

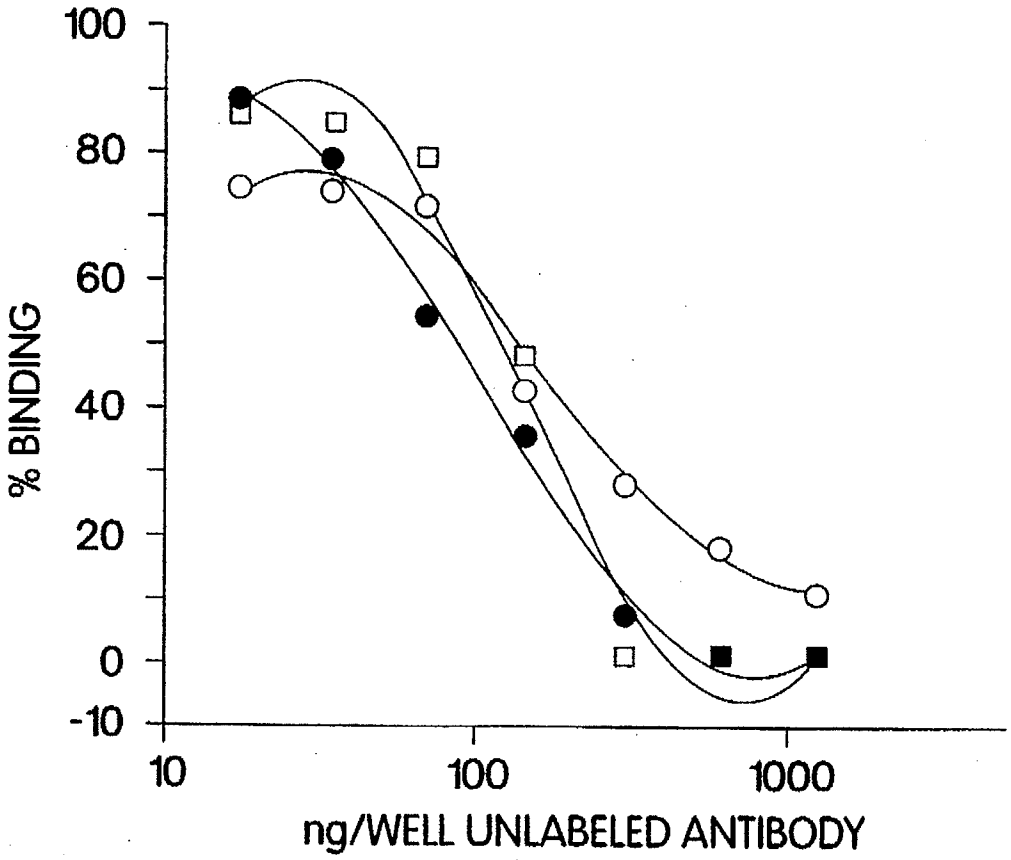
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Fig. 7

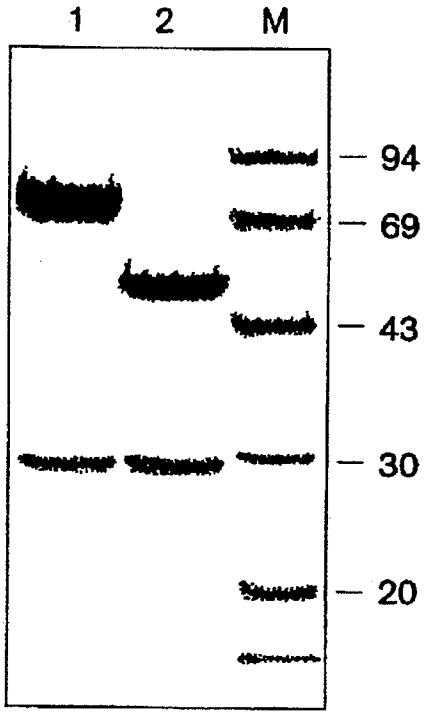

Fig. 8A

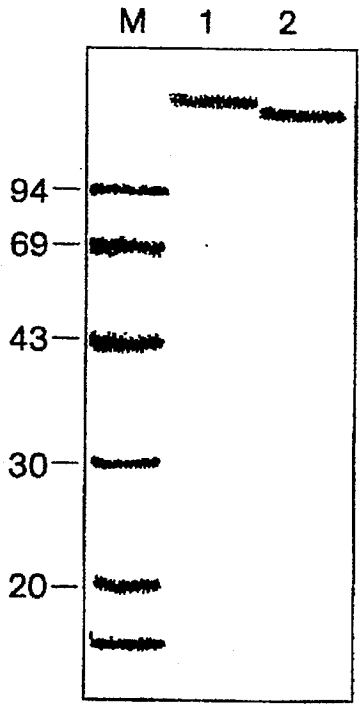

Fig. 8B

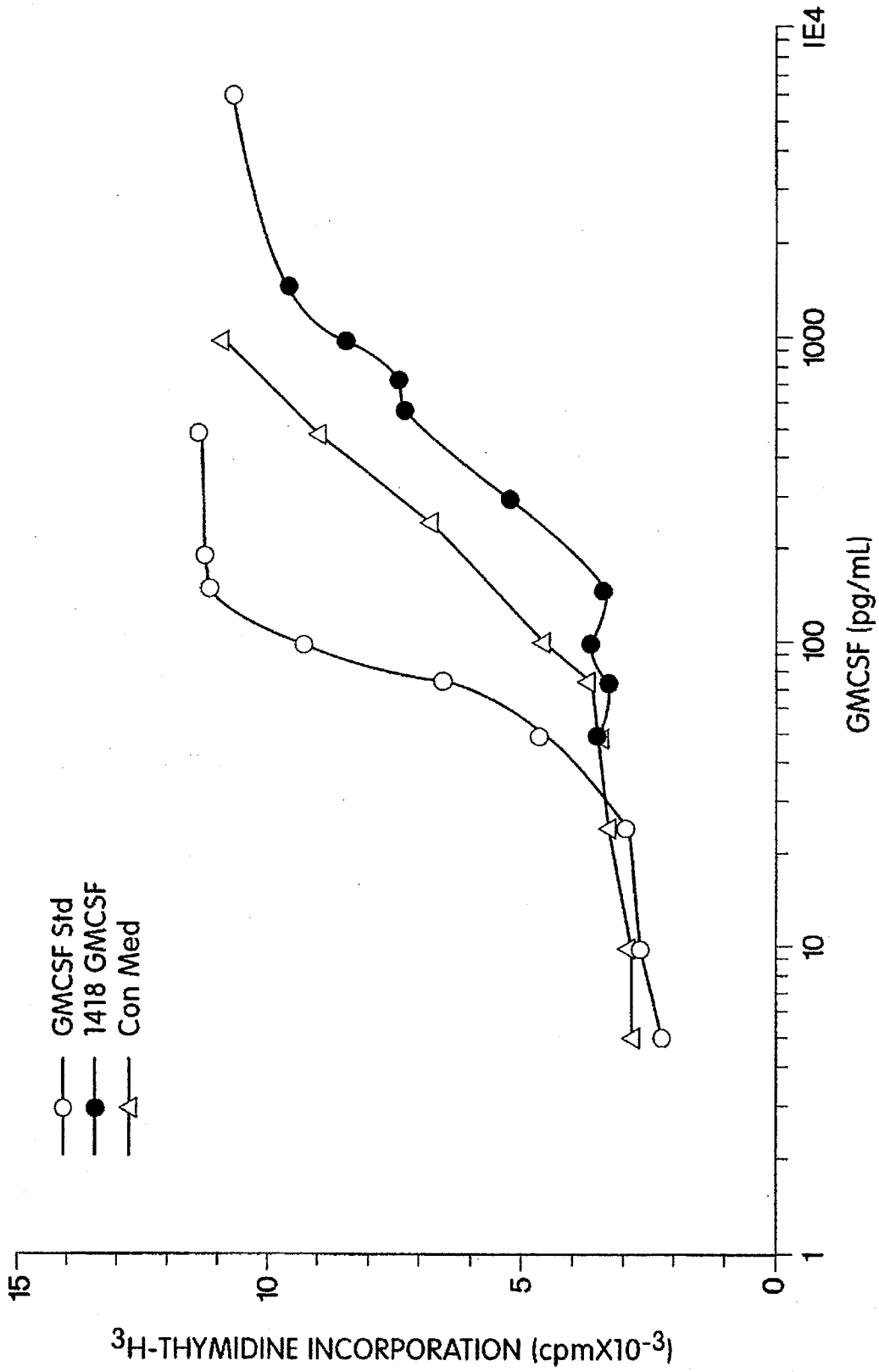
GMCSF Std
1418 GMCSF
Con Med
15
10
5
0
1
10
100
1000
IE4
GMCSF (pg/mL)
^{3}H-THYMIDINE INCORPORATION (cpmX10^{-3})

Fig. 9

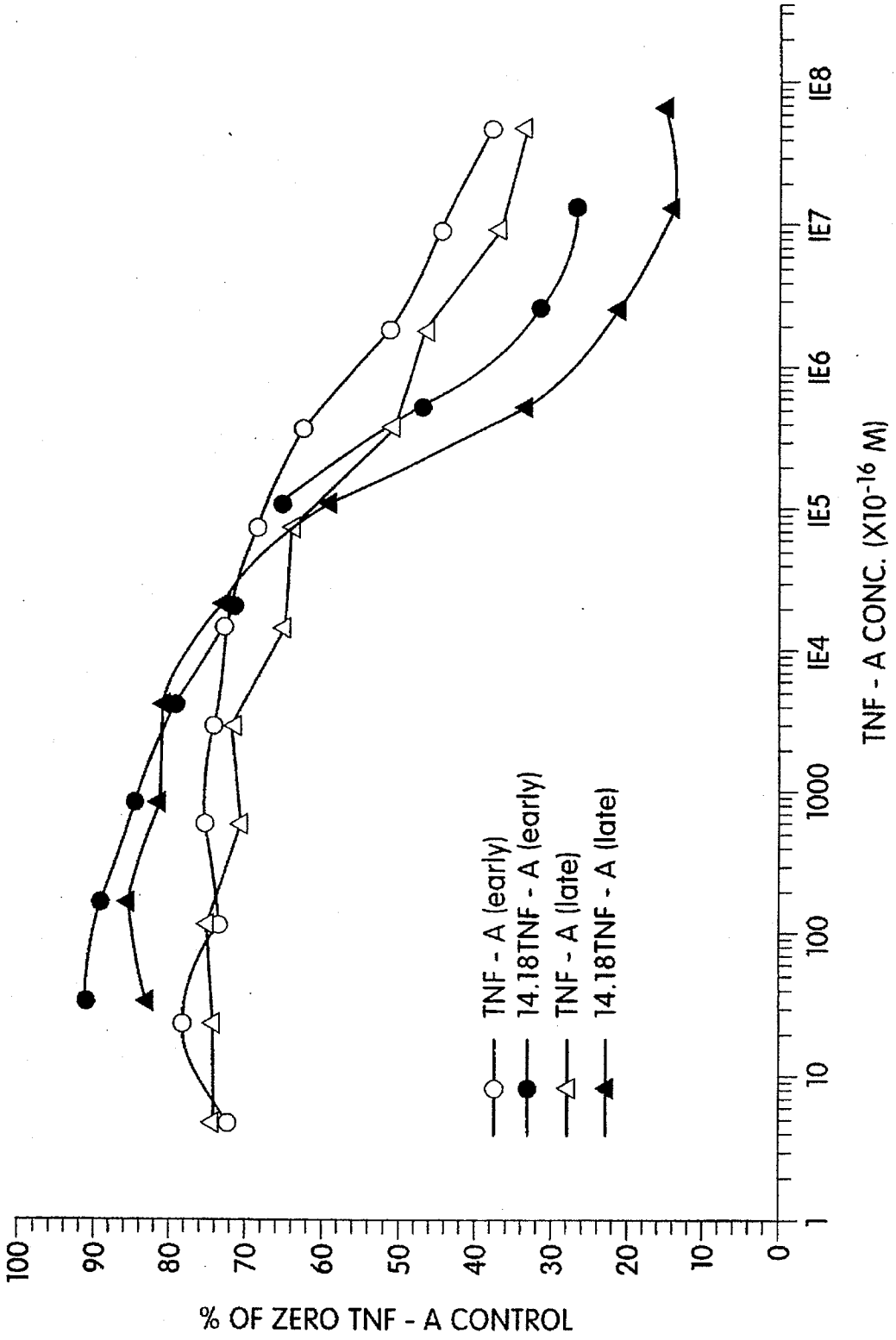
% OF ZERO TNF - A CONTROL
100
90
80
70
60
50
40
30
20
10
0
TNF - A CONC. (X10-16 M)
1
10
100
1000
IE4
IE5
IE6
IE7
IE8
TNF - A (early)
14.18TNF - A (early)
TNF - A (late)
14.18TNF - A (late)

Fig. 10

RECOMBINANT ANTIBODY CYTOKINE FUSION PROTEINS

This is a continuation of application Ser. No. 07/788,765 filed Nov. 7, 1991 (now abandoned), which is a continuation-in-part of application Ser. No. 07/612,099, filed Nov. 9, 1990 (now abandoned), the disclosures of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

The present invention relates generally to therapies involving the selective destruction of cells in vivo and to compositions of matter useful in the treatment of various cancers and vital infections. In particular, this invention relates to genetically engineered antibody fusion constructs capable of targeting an infected cell, and eliciting a localized inflammatory response such that the cell is killed or neutralized.

Tumor necrosis factor (TNFα) and lymphotoxin (LT or TNFβ) were first identified on the basis of their ability to directly kill certain tumors. However, many other biological activities are now attributed to these closely related cytokines. These include effects on a variety of cell types, such as the induction of histocompatibility antigens and adhesion receptors, as well as those resulting in inflammation, vascular permeability changes and mononuclear cell infiltration (Goeddel, D. V. et al. (1986) Symp. Quant. Biol. 51:597, Cold Spring Harbor; Beutler, B. and Cerami, A. (1988) Ann. Rev. Biochem. 57:505; Paul N. L. and Ruddle, N. H. (1988) Ann. Rev. Immunol. 6:407). The very short half-life of both TNFα and LT ensures that these inflammatory reactions do not occur systematically, but only at the sites of release from TNF-producing cells.

This ability to elicit a localized inflammatory response could be used in the treatment of solid tumors or other diseased tissue. For example, if it were possible to specifically deliver either TNFα or LT to a tumor site, a local inflammatory response could lead to an influx of effector cells such as natural killer cells, large granular lymphocytes, and eosinophils, i.e., the cells that are needed for antibody-dependent cellular cytotoxicity (ADCC) activity.

A way to deliver the lymphokine to a specific site in vivo is to conjugate it to an immunoglobulin specific for the site. However, the fusion of protein domains to the carboxy-termini of immunoglobulin chains or fragments can have unexpected consequences for the activities of both the protein to be fused and the immunoglobulin, particularly as far as antigen binding, assembly and effector functions are concerned. For example, the desired biological functions of the individual proteins may not be maintained in the final product.

Another potential problem with expressing proteins, such as the lymphokine LT, as a fusion protein to an immunoglobulin chain is that the native molecule exists in solution as a trimer and binds more efficiently to its receptor in this form. Thus, it seems unlikely that trimerization could still occur when LT is attached to an immunoglobulin heavy (H) chain via amino terminus and is assembled into an intact Ig molecule containing two paired H chain fusion polypeptides. Secondly, the ability of the fused LT to bind its receptor may be severely compromised if a free amino terminus is required for receptor binding activity. In fact, it has been postulated that the amino and carboxy-termini of TNFα, and, by analogy, LT, together form a structure that is required for receptor interaction.

It is an object of the invention to provide compositions of matter capable of selectively destroying cells in vivo, and

therapeutic methods for accomplishing this. It is also an object of the invention to provide compositions of matter and therapeutic methods for selectively delivering a cytokine to a target cell for the purpose of destroying the target cell either directly or by creating an environment lethal to the target cell.

SUMMARY OF THE INVENTION

This invention relates to immunoconjugates which include an immunoglobulin (Ig), typically a heavy chain, and a cytokine, and to the use of the immunoconjugates to treat disease. The immunoconjugates retain the antigen-binding activity of the Ig and the biological activity of the cytokine and can be used to specifically deliver the cytokine to the target cell.

The term "cytokine" is used herein to describe proteins, analogs thereof, and fragments thereof which are produced and excreted by a cell, and which elicit a specific response in a cell which has a receptor for that cytokine. Preferable cytokines include the interleukins such as interleukin-2 (IL-2), hematopoietic factors such as granulocyte-macrophage colony stimulating factor (GM-CSF), and tumor necrosis factor alpha (TNFα).

The term "lymphokine" as used herein describes proteins, analogs thereof, and fragments thereon produced by activated lymphocytes, and having the ability to elicit a specific response in a cell which has a receptor for that lymphokine, e.g., lymphotoxins. Lymphokines are a particular type of cytokine.

In preferred embodiments, the immunoconjugate comprises a chimeric Ig chain having a variable region specific for a target antigen and a constant region linked through a peptide bond at the carboxy terminus of the heavy chain to the cytokine.

Immunoconjugates of the invention may be considered chimeric by virtue of two aspects of their structure. First, the immunoconjugate is chimeric in that it includes an immunoglobulin chain (typically but not exclusively a heavy chain) of appropriate antigen binding specificity fused to a given cytokine. Second, an immunoconjugate of the invention may be chimeric in the sense that it includes a variable region and a constant region which may be the constant region normally associated with the variable region, or a different one and thus a V/C chimera; e.g., variable and constant regions from different naturally occurring antibody molecules or from different species. Also embraced within the term "immunoconjugate" are constructs having a binding domain comprising framework regions and variable regions (i.e., complementarity determining regions) from different species, such as are disclosed by Greg Winter et al., GB2, 188, 638. Preferably, the cytokine of the immunoconjugate can be a protein which naturally forms a dimeric or multimeric structure when unfused, such as LT or TNFα.

In a preferred embodiment, the chimeric Ig chain comprises a heavy (H) chain which includes the CH1, CH2 and CH3 domains. A proteolytic cleavage site may be located between the Ig heavy chain and the cytokine so that, when the conjugate reaches the target cell, the cytokine is cleaved from the heavy chain. A "proteolytic cleavage site" is an amino acid sequence recognizable by a protease with cleaves either within or proximal to the sequence. Preferably, the variable region is derived from a mouse (i.e. its DNA sequence or its amino acid sequence is based on a DNA or amino acid sequence of mouse origin) and the constant region (preferably including the framework region amino acids of the variable region) is derived from a human;

and the variable region of the heavy chain is derived from an Ig specific for a virus-infected cell, or for a tumor-associated or viral antigen. Preferably, the chimeric Ig chain can be assembled into the immunoconjugate by combining it with an appropriate counterpart (light or heavy) chain to form a monovalent antigen-binding region, which can then be associated to produce a divalent immunoconjugate specific for the target antigen.

The invention also features DNA constructs encoding the above-described immunoconjugates, and cell lines, e.g., myelomas, transfected with these constructs.

The invention also includes a method of selectively delivering a cytokine to a target cell, which method includes providing a cytokine immunoconjugate including a chimeric Ig chain including an Ig heavy chain having a variable region specific for the target cell and a constant region joined at its carboxy terminus by a peptide bond to a cytokine, and an Ig light chain combined with the chimeric Ig heavy chain, forming a functional antigen-binding site, and administering the immunoconjugate in an amount sufficient to reach the target cell to a subject harboring the target cell.

The invention thus provides an immunoconjugate in which the antigen binding specificity and activity of an antibody are combined in one molecule with the potent biological activity of a cytokine. An immunoconjugate of the invention can be used to deliver selectively a cytokine to a target cell in vivo so that the cytokine can exert a localized biological effect, such as a local inflammatory response, stimulation of T cell growth and activation, and ADCC activity. Such conjugates, depending on their specificity and biological activity can be used to treat diseases involving viral infections, or cancer, by targeted cell lysis, according to methods of the invention.

DESCRIPTION OF THE DRAWINGS

The foregoing and other objects of the present invention, and the various features thereof, may be more fully understood from the following description, when read together with the accompanying drawings, in which:

FIG. **1** is a schematic representation of one embodiment of the immunoconjugate of the present invention;

FIGS. **2**A–D are diagrams of the construction of fusion proteins between LT and the human Ig H chain; wherein FIG. **2**A is a map of a human Cγ1 gene fragment cloned in plasmid pBR322; FIG. **2**B shows the Cγ1 gene fused to LT at the end of the CH2 domain; FIG. **2**C shows the Cγ1 gene fused to LT at the end of the CH3 domain; FIG. **2**D shows the cDNA encoding LT cloned in expression vector pDEM including promoter (arrow), the natural leader peptide of LT (open box), the first residue of the mature protein (+1) and mouse κ L-chain poly A and 3'untranslated sequence. Open boxes represent protein coding regions of Cγ1 in A–C; black boxes represent synthetic linkers used to join the protein coding sequences; and striped boxes represent LT coding sequences;

FIG. **3** is a strippled representation of an SDS-polyacrylamide gel showing an analysis of fusion protein chain assembly, wherein chimeric ch14.18 antibody is shown in lanes **1** and **4**; CH2-LT is shown in lanes **2** and **5**; and CH3-LT is shown in lanes **3** and **6**. The position of stained marker proteins and their apparent molecular weights are indicated. The dried gel was exposed to film for either 4 hr (lanes **1** and **4**) or 18 hr. Cells were labeled with ^{35}S-methionine and secreted proteins were precipitated with an anti-human κ antiserum and protein A and analyzed on an SDS gel either reduced (lanes **1–3**) or unreduced (lanes **4–6**);

FIGS. **4**A–B are graphs showing the comparison of LT cytolytic activities for native LT (Δ–Δ), CH2-LT (o--o) or CH3-LT (o--o, filled in) immunoconjugates. A sensitive clone of the mouse fibroblast line **929** was used in the 1-day assay with mitomycin C. Relative cell survival was quantitated by staining with crystal violet and measuring the absorbance at 630 nm. FIG. **4**A shows culture supernatants from transfected cells assayed after first quantitating the conjugates by ELISA. FIG. **4**B shows purified proteins assayed following protein A Sepharose or immunoaffinity chromatography;

FIG. **5** is a graph of the effect of pH during purification on the cytostatic activity of CH3-LT. The activities of native LT (o--o), CH3-LT in culture supernatant (Δ--Δ), CH3-LT purified by protein A Sepharose chromatography (¤--¤) and CH3-LT purified at pH 6.5 (Δ--Δ) were compared in the cytostatic assay (in the absence of mitomycin C) using a mouse **929** subclone;

FIGS. **6**A–B are graphs of the cytolytic and cytostatic activities of LT and CH3-LT GD2-positive M21 human melanoma cells. M21 cells were seeded in 96-well plates in the [Bpresence (FIG. **6**A) or absence (FIG. **6**B) of mitomycin C and dilutions of LT (o--o) or CH3-LT (o--o, filled in) were added. Relative cell growth was measured by staining wells with crystal violet after 48 hr and measuring the absorbance at 630 nm;

FIG. **7** is a graph of the antigen binding activity of Ig/LT immunoconjugates. Relative binding was determined in a competitive antigen binding assay using ch14.18 antibody conjugated to HRP as tracer and either unlabeled ch14.18 (o--o), CH2-LT (o--o, filled in) or labeled ch14.18 (¤--¤) as competitor.

FIGS. **8**A–B are strippled representations of an SDS-polyacrylamide gel showing an analysis, under reducing (R) or nonreducing (NR) conditions, of the fusion protein ch**14.18**-CH3-GM-CSF (lane **1**) and the unfused protein ch14.18 (lane **2**), where M is molecular weight markers of indicated sizes.

FIG. **9** is a graph of GM-CSF activity of the Ig/GM-CSF immunoconjugate ch**14.18**-GM-CSF (o--o, filled in) compared to a GM-CSF standard (o--o) and conditioned medium (Δ--Δ).

FIG. **10** is a graph of TNF∝ activity of the Ig/TNF immunoconjugatees ch**14.18**-TNF-∝ (early) (o--o, filled in), ch**14.18**-TNF-∝ (late) (Δ--Δ, filled in), compared to TNF-∝ (early) (o--o) and TNF-∝ (late) (Δ--Δ).

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to immunoconjugates useful for killing a malignant or virus-infected target cell. The immunoconjugate includes a conjugate of an antibody portion having a specificity for a surface antigen on a virus-infected or malignant cell, and a cytokine.

FIG. **1** shows a schematic view of a representative immunoconjugate **10**. In this embodiment, cytokine molecules **2** and **4** are peptide bonded to the carboxy termini **6** and **8** of CH3 regions **10** and **12** of antibody heavy chains **14** and **16**. V_L regions **26** and **28** are shown paired with V_H regions **18** and **20** in a typical IgG configuration, thereby providing two antigen binding sites **30** and **32** at the amino ends of immunoconjugate **10** and two cytokine receptor-binding sites **40** and **42** at the carboxy ends of immunoconjugate **10**. Of course, in their broader aspects, the immunoconjugates need not be paired as illustrated.

The immunoconjugates of this invention can be produced by genetic engineering techniques; i.e., by forming a nucleic

acid construct encoding the chimeric immunoconjugate. Preferably, the gene construct encoding the immunoconjugate of the invention includes, in 5' to 3' orientation, a DNA segment which encodes a heavy chain variable region, a DNA segment encoding the heavy chain constant region, and DNA coding for the cytokine. The fused gene is assembled in or inserted into an expression vector for transfection of the appropriate recipient cells where it is expressed. The hybrid chain can be combined with a light (or heavy) chain counterpart to form monovalent and divalent immunoconjugates.

The cytokine can be any cytokine or analog or fragment thereof which has a therapeutically valuable biological function. Useful cytokines include the interleukins and hematopoietic factors such as interleukin-2 (IL-2) and granulocyte-macrophage colony stimulating factor (GMCSF). Lymphokines such as LT and TNF∝, which require the formation of multimeric structures to function, can also be used. The gene encoding the lymphokine or cytokine can be cloned de novo, obtained from an available source, or synthesized by standard DNA synthesis from a known nucleotide sequence. For example, the DNA sequence of LT is known (see, e.g. Nedwin et al. (1985) Nucleic Acids Res. 13:6361), as are the sequences for interleukin-2 (see, e.g., Taniguchi et al. (1983) Nature 302:305–318), granulocyte-macrophage colony stimulating factor (see, e.g., Gasson et al. (1984) Science 266:1339–1342), and tumor necrosis factor alpha (see, e.g., Nedwin et al. 1. Ibid.)

The heavy chain constant region for the conjugates can be selected from any of the five isotypes: alpha, delta, epsilon, gamma or mu. Heavy chains or various subclasses (such as the IgG subclasses **1–4**) can be used. The light chains can have either a kappa or lambda constant chain. DNA sequences for these immunoglobulin regions are well known in the art. (See, e.g., Gillies et al. (1989) J. Immunol. Meth. 125:191).

In preferred embodiments, the variable region is derived from an antibody specific for the target antigen (an antigen associated with a diseased cell such as a cancer cell or virus-infected cell), and the constant region includes the CH1, CH2 and CH3 domains. The gene encoding the cytokine is joined, (e.g., by appropriate linkers, e.g., by DNA encoding $(Gly_4\text{-}Ser)_3$ in frame to the 3' end of the gene encoding the constant region (e.g., CH3 exon), either directly or through an intergenic region. In certain embodiments, the intergenic region can comprise a nucleotide sequence coding for a proteolytic cleavage site. This site, interposed between the immunoglobulin and the cytokine, can be designed to provide for proteolytic release of the cytokine at the target site. For example, it is well known that plasmin and trypsin cleave after lysine and arginine residues at sites that are accessible to the proteases. Many other site-specific endoproteases and the amino acid sequences they attack are well-known.

The nucleic acid construct can include the endogenous promoter and enhancer for the variable region-encoding gene to regulate expression of the chimeric immunoglobulin chain. For example, the variable region encoding genes can be obtained as DNA fragments comprising the leader peptide, the VJ gene (functionally rearranged variable (V) regions with joining (J) segment) for the light chain or VDJ gene for heavy chain, and the endogenous promoter and enhancer for these genes. Alternatively, the gene coding for the variable region can be obtained apart from endogenous regulatory elements and used in an expression vector which provides these elements.

Variable region genes can be obtained by standard DNA cloning procedures from cells that produce the desired antibody. Screening of the genomic library for a specific functionally rearranged variable region can be accomplished with the use of appropriate DNA probes such as DNA segments containing the J region DNA sequence and sequences downstream. Identification and confirmation of correct clones are then achieved by DNA sequencing of the cloned genes and comparison of the sequence to the corresponding sequence of the full length, properly spliced mRNA.

The target antigen can be a cell surface antigen of a tumor cell, a virus-infected cell or another diseased cell. Genes encoding appropriate variable regions can be obtained generally from Ig-producing lymphoid cells. For example, hybridoma cell lines producing Ig specific for tumor associated antigens or viral antigens can be produced by standard somatic cell hybridization techniques. (See, e.g., U.S. Pat. No. 4,96,265.) These Ig-producing cell lines provide the source of variable region genes in functionally rearranged form. The variable region genes will typically be of murine origin because this murine system lends itself to the production of a wide variety of Igs of desired specificity.

The DNA fragment containing the functionally rearranged variable region gene is linked to a DNA fragment containing the gene encoding the desired constant region (or a portion thereof). Ig constant regions (heavy and light chain) can be obtained from antibody-producing cells by standard gene cloning techniques. Genes for the two classes of human light chains and the five classes of human heavy chains have been cloned, and thus, constant regions of human origin are readily available from these clones.

The fused gene encoding the hybrid IgH chain is assembled or inserted into expression vectors for incorporation into a recipient cell. The introduction of gene construct into plasmid vectors can be accomplished by standard gene splicing procedures.

The chimeric IgH chain can be co-expressed in the same cell with a corresponding L chain so that a complete immunoglobulin can be expressed and assembled simultaneously. For this purpose, the heavy and light chain constructs can be placed in the same or separate vectors.

Recipient cell lines are generally lymphoid cells. The preferred recipient cell is a myeloma (or hybridoma). Myelomas can synthesize, assemble, and secrete immunoglobulins encoded by transfected genes and they can glycosylate protein. A particularly preferred recipient cell is the Sp2/0 myeloma which normally does not produce endogenous immunoglobulin. When transfected, the cell will produce only Ig encoded by the transfected gene constructs. Transfected myelomas can be grown in culture or in the peritoneum of mice where secreted immunoconjugate can be recovered from ascites fluid. Other lymphoid cells such as B lymphocytes can be used as recipient cells.

There are several methods for transfecting lymphoid cells with vectors containing the nucleic acid constructs encoding the chimeric Ig chain. A preferred way of introducing a vector into lymphoid cells is by spheroblast fusion. (see, Gillies et al. (1989) Biotechnol. 7:798–804). Alternative methods include electroporation or calcium phosphate precipitation.

Other useful methods of producing the immunoconjugates include the preparation of an RNA sequence encoding the construct and its translation in an appropriate in vivo or in vitro system.

The immunoconjugate of this invention can be used to deliver selectively a cytokine to a target cell in vivo so that the cytokine can exert a localized biological effect such as a

local inflammatory response, stimulation of T cell growth and activation, and ADCC activity. A therapeutically effective amount of the immunoconjugate is administered into the circulatory system of a subject harboring the target cell.

The invention is illustrated further by the following non-limiting Examples.

1. Plasmid Construction

Described below is the construction of PdHL2, a plasmid which contains the human Cγ1 heavy and kappa light chain gene sequences as well as insertion sites for V region cDNA cassettes (Gillies et al. (1989) J. Immunol. Meth. 125:191). This plasmid may be used as a starter plasmid for constructing any IgH chain cytokine fusion. For example, PdHL2 was used for the expression of Ig/LT fusion proteins. A LT cDNA was isolated from a human peripheral blood leukocyte library cloned in λgt10. The sequence was identical to that reported in the literature by Nedwin et al. (Nucleic Acids Res (1985) 13:6361). The cDNA was inserted into vector pDEM (Gillies et al., ibid) as an XhoI fragment after first removing most of the 3' untranslated region with Bal31 nuclease. The resulting plasmid, pDEM-LT (FIG. 2), expresses (in transfected cells) a fusion mRNA with a 5' untranslated sequence derived from the metallothionein (MT) promoter, the LT coding sequence and a 3' untranslated sequence and a poly A addition signal from the mouse Cκ gene. Fusion protein-encoding vectors were constructed by ligating HindIII to TaqI (CH2-LT) or HindIII to NsiI (CH3-LT) fragments of the human Cγ1 gene to HindIII and PvuII digested PDEM-LT using synthetic DNA linkers (FIG. 2). These linkers:

(5'-CGAAGAAAACCATCTCCAAA/CTCCCTGGTGTTGGCCTCAC ACCTTCAG-3' (for CH2-LT); and

5'-TGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCT GTCCCCGGGTAAA/CTCCCTGGTGTTGGCCTCACACCTTCAG-3')

provide the protein coding sequence from the unique site (NsiI or TaqI) to the end of the heavy-chain domain (indicated by the slash), and join them to the amino terminus of the mature form of LT (up to the unique PvuII site). The linker for the CH3 fusion protein also includes a silent mutation that creates a SmaI site close to the end of the domain for future use in constructing fusion proteins. The DNA sequences at the junction of each construct were confirmed and each HindIII to EcoRI fragment was inserted into plasmid pdHL2-VCγ1κ(14.18). This plasmid contains the V cassettes for the ch14.18 anti-ganglioside GD2 antibody (Gillies et al., ibid.).

2. Cell Culture and Transfection

Sp2/0 Ag14 mouse hybridoma cells were maintained and transfected as described by Gillies et al. (BioTechnology (1989) 7:8799). Drug selection in methotrexate (MTX) was initiated 24 hours after transfection by adding an equal volume of medium containing MTX at 0.1 μM. Two additional feedings with selection medium were done at 3 day intervals. Transfectants secreting human Ig determinants were identified by ELISA (Gillies et al., 1989. ibid), grown in medium containing increasing concentrations of MTX, and subcloned by limiting dilution in medium containing MTX at 5 μM.

3. Purification and Characterization of Fusion Proteins

Proteins were biosynthetically labeled by incubating transfected cells (1×10^6/mL) for 16 hr in growth medium containing ^{35}S-methionine (50 μCi/mL-Amersham). Culture supernatants were then clarified by centrifugation in a microcentrifuge and the labeled proteins were immmunoprecipitated with polyclonal anti-human κ chain antisera (Jackson Immunoresearch, Bar Harbor, Me.) and protein A Sepharose (Repligen, Corp., Cambridge, Mass.). Protein samples were boiled for 5 min. in gel sample buffer in the presence or absence of 2-mercaptoethanol and analyzed on a 7% polyacrylamide gel. Proteins were detected by fluorography (diphenyloxazole in DMSO) and autoradiography.

Unlabeled proteins were purified from spent suspension culture medium by either immunoaffinity chromatography with a monoclonal anti-human κ antibody for the CH2-LT protein or by protein A Sepharose chromatography for the CH3-LT protein. All materials were concentrated by membrane dialysis into PBS. An alternative procedure for purification of the CH3-LT protein was developed to prevent the loss of LT activity during elution from the protein A column. Spent culture media was diluted with three volumes of 10 mM sodium phosphate buffer (pH 6.5) and loaded onto a Bakerbond AbX (J. T. Baker) column at room temperature. The column was washed with 10 mM sodium phosphate buffer until the absorbance returned to baseline and then with PBS, pH 6.5 (150 mM NaCl, 10 mM sodium phosphate, pH 6.5). The CH3-LT protein was eluted with 150 mM NaCl, 50 mM sodium phosphate, pH 6.5.

4. Activity Assay

The antigen binding activity of the Ig-LT proteins was measured as described in Gillies et al. (J. Immunol. Meth. (1989) 125:191), and LT activity was determined in the cytolytic or cytostatic assay (Kahn et al. (1982)) utilizing the 159124T2.5 subclone of the mouse L929 cell line (provided by Dr. H. Schreiber, University of Chicago). Cells were seeded into 96-well plates at 4×10^4 cells per well, with (cytolytic) or without (cytostatic) mitomycin C (2 μg/mL), and 10 μL of the test sample was added after 24 hr. Cells were stained either 24 or 48 hr later (see FIG. descriptions) with crystal violet and the amounts of dye retained in the wells were compared to those of untreated wells and those receiving the LT standard (R&D Systems). The same assay was also carried out with the GD2-bearing human melanoma line M21, originally provided by D. L. Morton, University of California, Los Angeles. The latter cell line was also used for measuring CDC and ADCC activity as described earlier (Gillies et al. (1990) Human Antibody. Hybridomas 1:47)

5. Expression of Ig/LT Immunoconjugates

The Ig/LT immunoconjugates were made by directly fusing the cDNA sequence encoding the mature form of LT to the end of either the CH2 or CH3 exon of the human Cγ1 gene (FIG. 2) with the appropriate synthetic linkers. This gene fusion was then combined in a vector together with the V regions of murine antibody 14.18 and the human Cκ gene, and expressed in transfected Sp2/0 cells. These immunoconjugates were then expressed and tested for antigen binding activity and Ig chain assembly. The immunoconjugates retained antigen binding when measured in a competitive antigen binding ELISA (see below), and were assembled. Cells expressing these immunoconjugates were labeled with ^{35}S-methionine, and the secreted proteins were analyzed by SDS-PAGE in the presence or absence of reducing agent.

As seen in FIG. 3, the CH2-LT immunoconjugate was expressed as a mixture of whole (approximately 180 Kd) and half (90 Kd) molecules. The CH3-LT fusion protein, on the other hand, consisted entirely of fully assembled molecules. This result is not surprising since the CH3 domain is most responsible for Ig chain assembly. The reason why a portion of the CH2-LT did assemble, i.e. formed disulfide bonds in the hinge domain of the antibody, is likely due to the dimerization of the carboxy-terminal LT domains.

6. Biological Activity of Ig/LT Conjugates

The LT activities of the CH2-LT and CH3-LT conjugates were compared in the standard cytolytic assay (Kahn, A. et

al. (1982) "A standardized automated computer assisted micro-assay for lymphotoxin." In: *Human Lymphokines, Biological response modified*; (Kahn and Hill, eds.) Academic Press, New York, p. 23), using a mouse L929 subclone. This assay measures the ability of the immunoconjugate to bind to the TNF/LT receptor and trigger the active cell killing process in this cell line. When crude preparations (culture supernatants) were compared (FIG. 4A), CH3-LT was found to be much more active (nearly 100 fold by this assay) than CH2-LT and exhibited approximately the same specific activity per mole as the LT standard. This higher activity of CH3-LT is likely due to the increased proportion of fully assembled H-chain fusion proteins. Thus, the presence of the CH3 exon in the immunoconjugate may allow the H-chains to associate more efficiently, perhaps positioning the LT domains in a manner that allows for dimerization and, as a consequence, more LT receptor binding.

When purified preparations were compared, the difference in activities between CH2-LT and CH3-LT was still evident, but the activity of the conjugates, especially CH3-LT, was greatly reduced compared to the LT control (FIG. 4B). Since both proteins had been purified by using elution steps at acidic pH (i.e., less than pH4), the pH sensitivity of the culture supernatants was examined, and the LT activity was found to be very acid labile.

An alternative purification scheme was developed in which the pH was not reduced to below 6.5. The material from this preparation was compared to that purified by protein A, the original starting material, and the LT standard. The results of the LT cytostatic assay, in the absence of mitomycin C, shown in FIG. 5, demonstrate that full LT activity can be maintained during purification provided low pH is avoided. This assay was used to give a better dose response for the LT control and to demonstrate that the relationship between CH2-LT and CH3-LT is consistent for both assay systems. The same results were obtained in the cytolytic assay.

The results show that full activity (as measured by this assay) can be maintained when LT is fused to an Ig H chain. The fact that the LT amino terminus is covalently bound to the carboxy-terminus of the antibody apparently does not prevent LT receptor binding or the steps subsequent to binding that are required for activating the cell killing process.

7. Antigen Binding and Effector Functions of Ig/LT immunoconjugates

The antigen binding activity of the immunoconjugates was measured on antigen-coated plates in either a direct binding or competition assay format. In the direct binding assay antigen binding activity was found to be much higher than that of the control ch14.18 antibody. Since the source of the GD2 antigen was a crude membrane extract from neuroblastoma cells, it is possible that the TNF/LT receptor is present in the preparation and that binding of the conjugate through the LT domain is responsible for this increased activity. When antigen binding was measured in a competition assay, the conjugate was found to compete with the labeled ch14.18 antibody for antigen only slightly more efficiently than the unlabeled ch14.18 antibody (FIG. 7).

The results show that it is possible to combine the antigen binding activity of an anti-tumor cell antibody with the potent biological activity of a cytokine. The presence of the CH3 exon in the immunoconjugate results in complete H-chain assembly and, as a consequence, higher LT and effector activities. The assembly of H chains may likely result in LT dimerization.

In addition, a free amino terminus is not necessary for LT binding to its receptor since in the highly active CH3-LT immunoconjugate, the amino terminus of the LT domain is peptide bonded to the Ig H chain.

8. Construction and Expression of Ig/GM-CSF Immunoconjugates

Ig/GM-CSF conjugates were made by joining a nucleotide sequence encoding GM-CSF to a nucleotide sequence encoding an Ig heavy chain, such that the encoded protein includes a heavy chain fused via the carboxy terminus to GM-CSF. The construct was made as follows. The mature protein coding sequence of GM-CSF was linked to the end of the CH3 exon of the human $C\gamma_1$ gene using PdHDL2 and appropriate oligonucleotide linkers, as described above for the LT conjugate and according to procedures well-known in the art. Also as described above for LT conjugates, the Ig heavy chain GM-CSF fused gene was combined with the heavy chain V region gene of the 14.18 anti-GD2 heavy chain, and carried on the same vector as the human Cκ gene and the light chain V region gene of the 14.18 antibody. After transfection of the DNA into hybridoma cells and consequent expression of the H and L genes, a complete ch14.18 antibody with GM-CSF attached to the end of each H chain was produced. The fusion protein was purified from conditioned medium using adsorbtion to and elution from protein A Sepharose. The peak material was diafiltered using an Amicon stirred cell into PBS and concentrated to approximately 1 mg/mL.

The fusion protein was analyzed by electrophoresis on a 10% SDS-polyacrylamide gel (FIG. 8) under reducing (R) or non-reducing (NR) conditions and the proteins were visualized by staining with Coomassie Blue. Lane 1, ch14.18-CH3-GMCSF; Lane 2, ch14.18; M, molecular weight markers of the indicated sizes in kD. The relative molecular weight of the fused H chain of 75 kD in lane 1 (R) is consistent with a glycosylated GM-CSF (~25 kD) being fused to the H chain (50 kD). Note in the non-reduced lane 1 that the fusion protein is assembled into a single high molecular weight species of ~200 kD.

9. Biological Activity of Ig/GM-CSF Conjugates

The GM-CSF activity of the ch14.18-GM-CSF fusion protein was examined in a proliferation assay using the GM-CSF-dependent cell line AML-193 (human acute myelogenous leukemia) (obtained from Daniel Santoli, Wistar Institute, Philadelphia, Pa.). Cells are cultured for 2 days in serum-free medium containing insulin and transferrin (but no GM-CSF), at which time GM-CSF or fusion protein sample dilutions are added. After five more days, 5 μCi of ^{3}H-thymidine is added to each well and after 16 hr, the cells are harvested in 10% trichloroacetic acid. After 30 min. on ice the precipitated material is collected on GF/C filters, dried and counted by liquid scintillation.

In FIG. 9, the proliferation obtained with varying amounts of GM-CSF, conditioned medium containing the secreted fusion protein, or ch14.18-GM-CSF purified by protein A Sepharose are compared. The results show that significant GM-CSF activity is maintained once the molecule is fused to the H-chain but that the activity is either 20% (conditioned medium) or 10% that of (purified fusion protein) GM-CSF standard. Maximum incorporation was obtained with less than 10 ng/mL of the purified fusion protein (GM-CSF equivalents or 50 ng of total protein). This slight loss of activity is not likely to affect the utility of this fusion protein, especially if large amounts of ch14.18-GM-CSF accumulate at the site of solid tumors expressing the GD2 antigen.

The in vivo half-life of the immunoconjugate was determined by injecting mice (20 μg injected in the tail vein) with ch14.18-GM-CSF. Samples of blood were collected at the

indicated times and the amount of fusion protein in the serum was determined by ELISA. The capture antibody was a polyclonal goat anti-human IgG (Fc-specific) and the detecting antibody was a horseradish peroxidase-conjugated goat anti-human K. As seen in Table 1, the half-life (calculated between the 24 hr and 4 day time points) was nearly 3 days. This compares to the published value of 85 min. in humans (Herrmann et al. (1989) J. Clin Oncol. 7:159–167). This increased half-life may compensate for the reduced activity of the fusion protein, especially since the local concentration of the immunoconjugate at the tumor site is likely to be increased by antibody targeting.

TABLE 1

Serum Concentration of ch14.18-CH3-GM-CS

Time after injection	Ab Concentration (ng/mL
4 hr	9210
16 hr	9660
24 hr	5950
4 days	2530

Mice were injected with 20 μg of the ch14.18-CH3-CSF fusion protein in the tail vein. Small samples (~50 μL) were taken from the tail vein and assayed for human antibody determinants.

10. Construction, Expression, and Activity of Ig/TNF Immunoconjugates

Ig/TNF immunoconjugates were made by fusing nucleotide sequences encoding TNFα and immunoglobulin heavy chain such that TNFα is fused to the carboxy terminus of the heavy chain. Briefly, the mature TNFα coding sequence was fused to the end of the human Cγ1 CH3 exon using oligonucleotides. The recombined fragment was joined downstream of the heavy chain V region encoding gene from the anti-GD2 mouse antibody 14.18; also contained in this vector was the human k gene, including both the V region gene encoding the light chain V region from the anti-GD2 mouse antibody 14.18 and the C region encoding gene. Hybridoma cells were transfected and selected as described above. Clones secreting human antibody determinants were expanded and used for the production and purification of the ch14.18-CH3-TNFα fusion protein by protein A Sepharose chromatography. The activity of the fusion protein was tested as described above for the CH3-LT fusion proteins.

As seen in FIG. **10**, the amount of cytotoxicity obtained with the fusion protein met or exceeded that of native TNFα at either early (20 hr) or late (24 hr) points in the assay. This fusion protein appears to be fully functional with respect to TNFα activity, even though it was purified using protein A Sepharose. The CH3-LT construct was partially inactivated by the elution at acidic pH using the same protocol.

The results described above for the Ig/LT, Ig/GM-CSF, and Ig/TNFα immunoconjugates demonstrate that an antibody can be genetically fused to a cytokine without the loss of antigen binding activity and effector functions of the antibody, or the receptor binding and biological activity of a cytokine.

11. Dosage

Immunoconjugates of the invention may be administered at a therapeutically effective dosage within the range of lug-100 mg/kg body weight per day. The immunoconjugate may be administered in physiologic saline or any other biologically compatible buffered solution. This solution may be administered systemically (e.g., by injection intravenously or intramuscularly).

Other Embodiments

The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The present embodiments are therefore considered to be in all respects as illustrative and not restrictive, the scope of the invention being indicated by the appended claims rather than by the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.

What is claimed is:

1. A recombinant immunoglobulin (Ig) chain comprising:

an Ig heavy chain and a cytokine which elicits a cytokine-specific biological response by a cell bearing a receptor for said cytokine, said Ig heavy chain comprising an N-terminal variable region specific for a cell bearing a cell surface antigen; and, CH1 and CH2 domains, said Ig heavy chain being joined at its carboxy-terminus by a peptide bond to the amino terminal amino acid of the cytokine.

2. The recombinant Ig chain of claim **1**, wherein the Ig heavy chain further comprises the CH3 domain.

3. The recombinant Ig chain of claim **1**, wherein a proteolytic cleavage site is located between the Ig heavy chain and the cytokine.

4. The recombinant Ig chain of claim **1**, wherein the variable region is a mouse Ig variable region and the CH1 and CH2 domains are human Ig CH1 and CH2 domains.

5. The recombinant Ig chain of claim **1**, wherein the variable region of the Ig heavy chain is specific for a cell surface antigen of a cancer cell or a virus-infected cell.

6. The recombinant Ig chain of claim **5**, wherein said cell surface antigen is a tumor associated antigen or a viral antigen.

7. The recombinant Ig chain of claim **1**, wherein the cytokine is tumor necrosis factor alpha.

8. The recombinant Ig chain of claim **1**, wherein the cytokine is interleukin-2.

9. The recombinant Ig chain of claim **1**, wherein the cytokine is a lymphokine.

10. The recombinant Ig chain of claim **9**, wherein the lymphokine is a lymphotoxin.

11. The recombinant Ig chain of claim **9**, wherein the lymphokine is granulocyte-macrophage colony stimulating factor.

12. The recombinant Ig chain of claim **9**, wherein the lymphokine is a lymphokine which forms a dimeric or multimeric structure.

13. A cytokine immunoconjugate comprising:

(a) a recombinant immunoglobulin (Ig) heavy chain having an N-terminal variable region specific for a cell bearing a cell surface antigen of a cancer cell or a virus-infected cell, a constant region comprising CH1 and CH2 domains, and a cytokine joined to the carboxy-terminus of said constant region by a peptide bond, wherein said cytokine elicits a cytokine-specific biological response by a cell bearing a receptor for said cytokine; and

(b) an Ig light chain having a variable region specific for said cell surface antigen, said Ig heavy and light chains forming together a functional antigen-binding site, such that said immunoconjugate displays both antigen-binding specificity and cytokine biological activity.

14. The immunoconjugate of claim **13**, wherein the recombinant Ig heavy chain has a constant region further comprising a CH3 domain.

15. The immunoconjugate of claim **13**, wherein the cytokine is interleukin-2.

16. The immunoconjugate of claim 13, wherein the cytokine is tumor necrosis factor alpha.

17. The immunoconjugate of claim 13, wherein the cytokine is a lymphokine.

18. The immunoconjugate of claim 17, wherein the lymphokine is lymphotoxin.

19. The immunoconjugate of claim 17, wherein the lymphokine is granulocyte-macrophage stimulating factor.

20. The recombinant Ig heavy chain of claim 1 or 13 wherein said cytokine elicits a cytotoxic response by cells bearing a receptor for said cytokine.

21. The recombinant Ig heavy chain of claim 1 or 13 wherein said cytokine elicits a proliferative response by cells bearing a receptor for said cytokine.

* * * * *

APPENDIX 3

RELATED PROCEEDINGS APPENDIX

NONE